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Biochemical Correlates of Progesterone-Induced Plasma Membrane Depolarization During the First Meiotic Division in *Rana* Oocytes

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Summary. Changes in protein synthesis, protein phosphorylation and lipid phosphorylation in the amphibian oocyte plasma membrane have been correlated with electrical changes following steroid induction of the completion of the first meiotic division. The oocyte first depolarizes from about −60 mV (inside negative) to about −25 mV 1 to 2 hr before breakdown of the large nucleus followed by a further depolarization beginning 3 to 6 hr after nuclear breakdown. The initial depolarization is associated with appearance of previously described cycloheximide-sensitive cytoplasmic factor(s) which induce both nuclear breakdown and plasma membrane depolarization. We found a similar ED_{50} (0.4 μM) for cycloheximide inhibition of nuclear breakdown, membrane depolarization, and [^{3}H]-leucine incorporation. Emetine (1 nM to 1 mM) was inactive. The period of cycloheximide sensitivity (first 5 hr) is essentially the same for plasma membrane depolarization and nuclear breakdown. The onset of the second depolarization phase following nuclear breakdown is associated with a marked increase in the rate of [^{3}H]-leucine and [$^{32}PO_4$] incorporation into membrane protein and lipid. Polyacrylamide gel electrophoresis of membrane protein and lipoprotein indicated that a major newly synthesized membrane component is proteolipid. An increase in [$^{32}PO_4$] incorporation into membrane phosphatidylserine and phosphatidylethanolamine (with a decrease in phosphatidylcholine [$^{32}PO_4$] begins during the second depolarization phase and coincides with the appearance of excitability in the oocyte plasma membrane. In toto, the bulk of the biochemical changes (proteins, phosphoproteins, proteolipids, phospholipids) appear to be associated with plasma membrane components and coincide with stepwise changes in membrane permeability to specific ions (e.g. Cl^-).

Key Words meiosis · plasma membrane · depolarization · phospholipid · proteolipid · phosphorylation · calcium

Introduction

The vertebrate oocyte is blocked in first meiotic prophase until the hormonal stimulus prior to ovulation. In oocytes of *Rana* a stepwise metabolic activation follows the hormonal release of the prophase block. Ca^{2+} is released from the oocyte surface within the first 1 to 2 min [22] and is followed by a fall in intracellular cAMP 10 to 15 min later [18, 30]. There is a transient rise in intracellular cGMP 1 to 2 hr after hormonal stimulation [6] followed by a transient increase in [^{3}H]-uridine incorporation at 3 to 5 hr [19]. The transient apparent increase in RNA synthesis is temporally associated with a requirement for protein synthesis and the appearance of a cytoplasmic factor called "maturation promoting factor" [12]. Eight to 12 hr after the hormonal stimulus, the large nucleus (germinal vesicle) rises to the surface of the animal hemisphere and the nuclear membrane breaks down. About the time of nuclear breakdown, there is a further increase in protein synthesis [19, 29, 39] and the oocyte plasma membrane undergoes a slow depolarization [41]. By the completion of the first meiotic division (20 to 24 hr) the *Rana* oocyte cytoplasm is essentially isopotential with the external medium [41] and the oocyte is now activatable [1]. A similar membrane depolarization is seen in *Xenopus* oocytes [5, 9, 14, 33] and increased protein phosphorylation has been reported to coincide with nuclear breakdown [10].

The present study examines some of the biochemical events which accompany stepwise changes in membrane properties during the first meiotic division. We report here in detail the time course of changes in membrane potential, as correlated with protein phosphorylation, phospholipid phosphate incorporation, phosphatidopeptide synthesis, and the dependence of these events on protein synthesis in oocytes of *Rana* as they undergo the first meiotic division induced by progesterone. We report that a stepwise depolarization of the oocyte plasma membrane occurs during the first meiotic division and that specific biochemical events appear to be associated with each step.

Materials and Methods

Materials

Sexually mature *Rana pipiens* females were obtained from the Lake Champlain region of the United States and maintained in artificial hibernation at 4° C. Oocytes free of follicular envelopes and follicle cells were prepared by the method of Masui

[12] with the modifications described elsewhere [41]. The Ringer's solution used throughout these studies is a modified amphibian Ringer's solution containing (in mM) 111 NaCl, 1.9 KCl, 1.1 $CaCl_2$, 2.4 $NaHCO_3$, 0.08 NaH_2PO_4 and 0.8 $MgSO_4$.

Progesterone (Steraloids, Pawling, N.Y.) was prepared in 95% ethanol, and 1 µl was added per ml of Ringer's solution. [^{3}H]-leucine, L-[3, 4, 5(N)] (>110 Ci/mmol), [^{45}Ca] (40 to 50 Ci/g) and [$^{32}PO_4$] (50 to 1,000 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). Cycloheximide and emetine were obtained from Sigma Chemical Co., St. Louis, Mo.

Meiotic Agonists: Nuclear Breakdown

Twenty-five denuded oocytes sample were incubated at 20 to 22 °C in 10 ml Ringer's solution containing progesterone and 0.03% ethanol as indicated. Untreated controls included oocytes in Ringer's solution as well as Ringer's solution with 0.04% ethanol. Except as noted, progesterone was present continuously during the period shown. Nuclear breakdown was detected by dissection of oocytes after boiling in 70% ethanol for 2 min. Unless otherwise noted, the percentage of nuclear breakdown was measured after 24-hr exposure.

Electrophysiological Measurements

Membrane potential measurements were made using a W-P Instruments M-707 microprobe system (New Haven, Conn.) and standard 2.5 M KCl-filled glass micropipettes [20, 41]. Oocytes were voltage clamped as described elsewhere [37]. Membrane potentials were usually stable within seconds after entry and were monitored for at least 30 to 60 sec in each oocyte.

Pulse Labeling with $^{32}PO_4$, $^{45}Ca^{2+}$ and/or [^{3}H]-Leucine

Pulse labeling was carried out as follows: oocytes were preincubated for 1 to 12 hr in hormone-containing or control media, then removed and placed in second vials containing the same medium plus $^{45}Ca^{2+}$, [^{3}H]-L-leucine and/or [$^{32}PO_4$] for 60 min. The oocytes were rinsed for 5 to 6 sec in Ringer's solution and counted (^{45}Ca) or homogenized in 2.0 ml of distilled water at ice-bath temperatures ([^{3}H], [$^{32}PO_4$]). 1.5 ml of the homogenate was removed for lipid and phosphoprotein extraction, 0.1 ml for total radioactivity cpm, and 0.2 ml was precipitated with 6% trichloroacetic acid (TCA) with 0.25% inorganic phosphate at 4 °C for 30 min. The TCA precipitate was collected on GFC glass fiber filters (Reeve Angel, Clifton, N.J.), washed four times with cold 6% TCA, and transferred to a scintillation vial for counting. Aliquots of the TCA filtrate were also taken for assay of the acid-soluble pool. When [^{3}H] or [^{32}P] was added to homogenates, less than 1% was recovered with the TCA precipitate. [^{32}P] and/or [^{3}H] were measured using an Intertechnique scintillation spectrometer and counts were corrected for quenching using an external standard. Care was taken to prevent either isotope or metabolite from becoming rate limiting. In general, less than 5% of the isotope in the medium was taken up by oocytes during the pulse-labeling periods shown.

Isolation of Plasma-Vitelline Membrane Complex

The isolation of the membrane complex has been described elsewhere [7, 8, 37]. Denuded oocytes are transferred to 3.0 ml of 0.24 M sucrose containing 1.1 mM $CaCl_2$ and 10 mM HEPES buffer (pH 7.0) at 20 to 22 °C. The oocytes are opened with fine-tipped forceps and the plasma-vitelline membrane complex everted by being drawn into a Pasteur pipette. Movement of the membrane complex in and out of the pipette results in a rapid displacement of residual platelets and granules and the clear membrane complex is then passed through two 3.0 ml volumes of buffered sucrose solution and extracted and/or counted.

Isolation of Phospholipids and Phosphatidopeptides

One volume of tissue was homogenized with five volumes of $CHCl_3/CH_3OH$ (1:1, vol/vol) and 0.50 volumes of aqueous 2 M KCl [4] and centrifuged. In the resulting biphasic system, the lower phase contains essentially all of the tissue lipids and proteolipids, including the polyphosphoinositides. An aliquot of the lower phase was taken to dryness under N_2, resuspended in $CHCl_3/CH_3OH$ (2:1) and subjected to two-dimensional thin-layer chromatography using the method of Skidmore and Entenman [28]. The individual marker phosphatides were visualized with I_2 vapor, scraped from the plate, eluted, counted and analyzed for lipid phosphorus [28]. The lower phase was collected and dialyzed against 10 times its volume of $CHCl_3/CH_3OH/HCl$ (2:1:0.01) in the dark at 4 °C until 85% or more of the starting solutes were dialyzed out [4]. Dialysis was then continued for at least five changes of neutral $CHCl_3/CH_3OH$ (1:1) and the retentate was recovered by taking to dryness in a flash evaporator. The residue was dissolved in an SDS-buffer and subjected to PAGE (*see below*).

Polyacrylamide Gel Electrophoresis (PAGE)

Oocyte protein was solubilized and reduced with 40 mM dithiothreitol in the presence of 1.0 mM EDTA and protease inhibitor (phenylmethylsulfonyl fluoride, 1.0 mM) in 20% Bio-Phore SDS buffer (Bio-Rad Laboratories, Richmond, Calif.) at pH 7.5 overnight at 37 °C. The solubilized protein was applied to $7^1/_2$% polyacrylamide gels (0.5 × 9 cm) prepared in the presence of 0.1% SDS and run at 6 to 7 mA per gel. The gels were stained with Coomassie blue R-250 and/or cut into 1.0 mm slices, solubilized with 30% H_2O_2, and counted. SDS-PAGE standards from Bio-Rad Laboratories were run routinely.

Results

Changes in Oocyte Plasma Membrane Potential and Conductance Correlated with Nuclear Breakdown and Completion of the First Meiotic Division

The time course of plasma membrane potential change and nuclear (germinal vesicle) breakdown (GVBD) in denuded oocytes of *Rana* is illustrated in Fig. 1. Oocytes were exposed to 32 µM progesterone for 20 min, then transferred to Ringer's solution; samples were taken at the times indicated. Values for membrane potential and nuclear breakdown over the first 9 hr were from a highly syn-

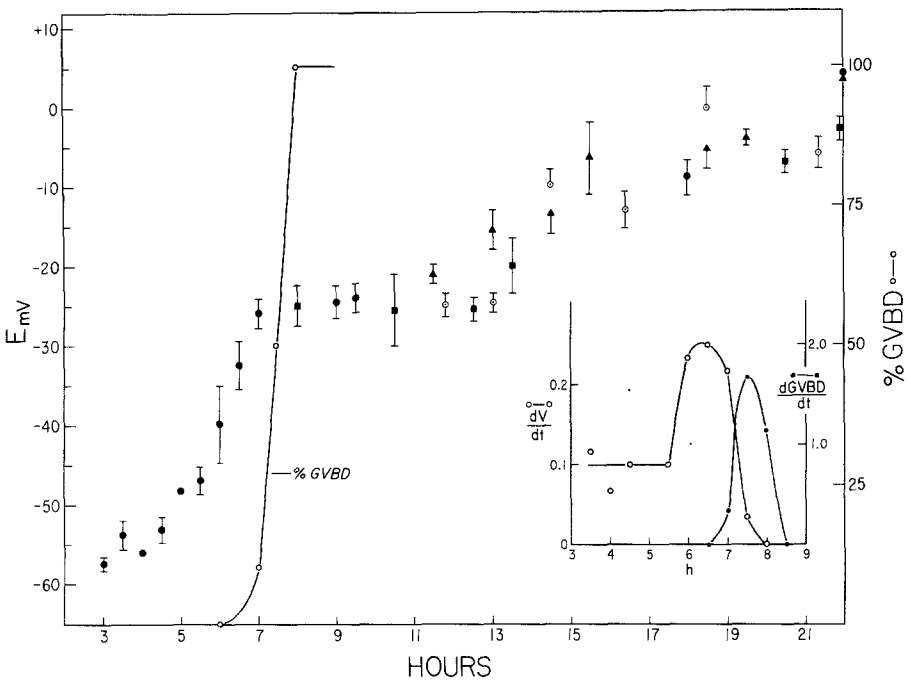

Fig. 1. Changes in plasma membrane potential as a function of time after exposure to inducing levels of progesterone (36 μM/20 min). Symbols indicate values for 4 to 6 sibling oocytes (±SEM) from four *Rana* females (o, •, ▲, ■). Time course of nuclear (germinal vesicle) breakdown (% GVBD) is shown for oocytes from female (•). Inset compares rate of change for nuclear breakdown with that for membrane potential for female (•) from 3 to 9 hr

chronous oocyte population from a single female and the inset compares dV/dt with dGVBD$/dt$ for this population. Membrane potentials from 10 to 22 hr are shown for oocyte populations from four females and in all cases nuclear breakdown was complete by 10 to 11 hr. Typically, depolarization is biphasic. The onset of depolarization preceded nuclear breakdown by 1 to $1\frac{1}{2}$ hr and approached a new steady-state potential by completion of nuclear breakdown. In experiments with six additional females, nuclear breakdown began as early as 6 hr and as late as 11 hr but onset of plasma membrane depolarization always preceded nuclear breakdown by 1 to 3 hr. As also shown, a second depolarization occurs 3 to 6 hr after completion of nuclear breakdown with the oocyte cytoplasm becoming essentially isopotential with Ringer's solution by the end of the first meiotic division (20 to 24 hr).

A comparison of current-voltage relationships (Fig. 2) indicates that membrane conductance decreases during the first depolarization phase (e.g. 4.7 to 2.2 μS), and again during the second depolarization phase (e.g. to 0.25 μS). Following the second depolarization, the oocyte plasma membrane becomes electrically excitable, producing positive-going action potentials in response to depolarizing stimuli. As reported elsewhere [37], conductance of the prophase-arrested oocyte membrane was 4.4 ± 0.5 μS and decreased to 2.0 ± 0.4 μS in oocytes which had depolarized to -18 ± 1.9 mV.

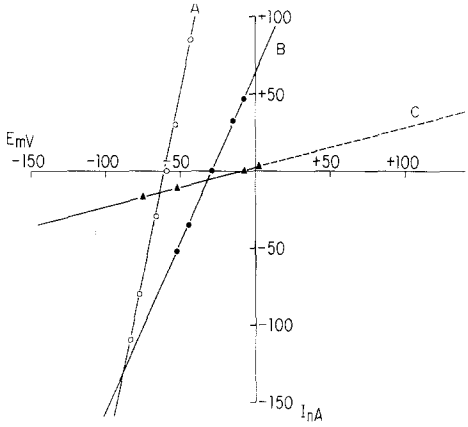

Fig. 2. Representative "instantaneous" current-voltage curves generated before the addition of progesterone (*A*), following nuclear breakdown (*B*), and near the completion of the first meiotic division (*C*). The dashed line indicates that the oocyte becomes excitable in this voltage range at this time

Effect of Cycloheximide on Protein Synthesis, Nuclear Breakdown and Plasma Membrane Depolarization

Previous studies have shown that addition of high concentrations (36 μM) of cycloheximide at the beginning of the induction period will block both nuclear breakdown and plasma membrane depolarization [41]. In Fig. 3 we compare the cycloheximide dose-dependence of [^{3}H]-leucine incorporation into protein with that for nuclear breakdown and plasma membrane depolarization. [^{3}H]-leucine incorporation decreased to about 25% of the

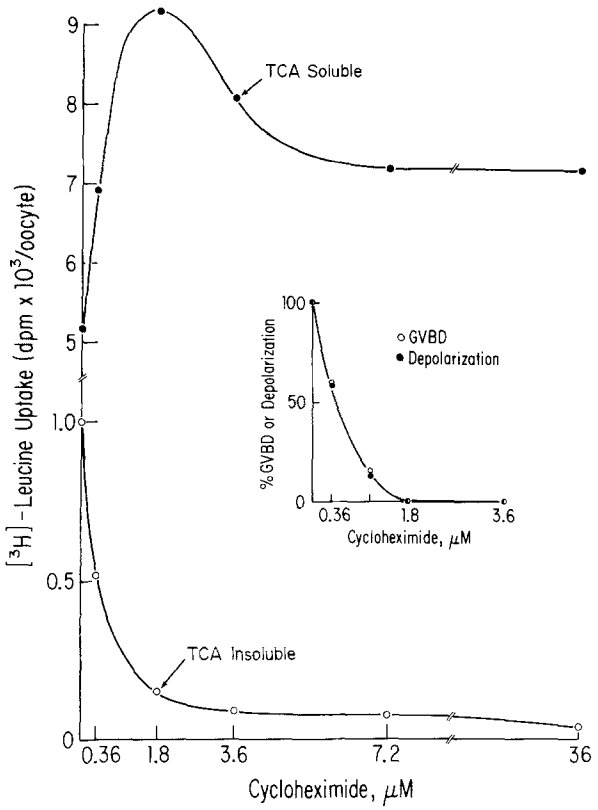

Fig. 3. [^{3}H]-leucine uptake and incorporation, membrane depolarization and nuclear breakdown (% GVBD) as a function of [cycloheximide]$_o$ in progesterone-treated denuded *Rana* oocytes. [^{3}H]-leucine uptake into the TCA-soluble phase (•—•) and TCA precipitate (o—o) was measured after 16 hr. Membrane potential and nuclear breakdown were measured after 16 to 18 and 24 hr, respectively, and expressed as % progesterone-treated control. GVBD was an all-or-nothing response, whereas oocytes underwent varying amounts of depolarization in the presence of progesterone and cycloheximide; depolarization is therefore expressed as percent of the progesterone-induced depolarization. Oocytes were exposed continuously to 1.6 μM progesterone in Ringer's solution at 20 °C

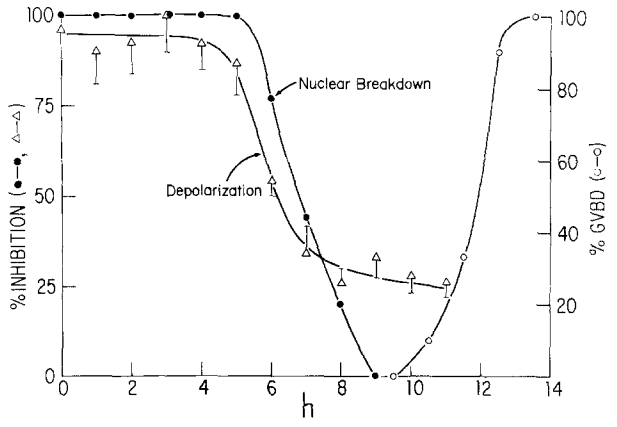

Fig. 4. Effect of adding cycloheximide (1.8 μM) at 1-hr intervals after exposure to 1.6 μM progesterone on both membrane potential and nuclear breakdown. Cycloheximide was added at the times indicated by △ (membrane potential) or • (nuclear breakdown). Membrane potential and nuclear breakdown were measured 17 to 20 and 24 hr after exposure to progesterone, respectively. % inhibition was calculated as $E_c - E_x / E_c \times 100$ where E_c is that for progesterone and cycloheximide treatment. The right-hand ordinate (o—o) indicates the time course of nuclear breakdown for sibling oocytes following exposure to 1.6 μM progesterone (continuous). Bars represent mean ± SEM for 4 to 6 sibling oocytes

progesterone-treated control with 1.8 μM cycloheximide and to about 5% of the control with 36 μM. Uptake of [^{3}H]-leucine into the acid-soluble pool peaked at low cycloheximide concentrations and remained above that of the control at higher cycloheximide concentrations. The inset in Fig. 3 illustrates the effect of [cycloheximide]$_o$ on nuclear breakdown and membrane depolarization in oocytes from the same animal. As shown, 50% inhibition of [^{3}H]-leucine incorporation, membrane depolarization and nuclear breakdown occurred at about 0.4 μM cycloheximide with complete inhibition of membrane depolarization and nuclear breakdown at 1.8 μM. Although not shown, emetine (10^{-9} to 10^{-3} M) had no effect on [^{3}H]-leucine incorporation, membrane potential or nuclear breakdown.

Time-Dependence of Plasma Membrane Depolarization and Nuclear Breakdown on Protein Synthesis

The critical period for the protein synthesis which is necessary for normal depolarization and/or nuclear breakdown was determined by adding 1.8 μM cycloheximide at 1-hr intervals after exposure to inducing levels of progesterone and examining the effect on both nuclear breakdown and plasma membrane depolarization. Membrane potential was measured 17 to 20 hr after the initial exposure to 1.6 μM progesterone, and nuclear membrane breakdown was measured 24 hr after initial exposure in cycloheximide-treated oocytes. The time course of nuclear breakdown was followed in progesterone-treated controls. Figure 4 illustrates the time course of nuclear breakdown (%GVBD) in control oocytes on the right-hand ordinate. The effects of cycloheximide are expressed on the left-hand ordinate as % inhibition of nuclear breakdown, and as % inhibition of membrane depolarization. In the experiment shown, nuclear breakdown began about 10 to 11 hr after exposure to progesterone and 50% breakdown occurred at about 12 hr. Addition of cycloheximide anytime during the first 5 hr completely blocked subsequent nuclear breakdown and membrane depolarization. Cycloheximide addition had a decreasing inhibitory effect when added after 6 to 8 hr, and was with-

Table 1. Effect of progesterone and cycloheximide on ^{45}Ca uptake by denuded *Rana* oocytes

Treatment	^{45}Ca Uptake, pmols/oocyte[a]
None	23.1
Cycloheximide, 1.8 μM	25.7
Progesterone, 1.6 μM	4.66
Progesterone, 1.6 μM + 1.8 μM cycloheximide	22.4

[a] 60-min pulse 8 to 9 hr after exposure to cycloheximide and/or progesterone. Values shown are for sibling oocytes and are representative of three such experiments. In the experiment shown, nuclear breakdown began 10 to 11 hr after exposure to progesterone.

out effect at 9 hr. In general, loss of inhibition followed the same time course for both nuclear breakdown and plasma membrane depolarization.

The depolarization prior to nuclear breakdown also appears to be associated with a decrease in Ca^{2+} uptake from the medium. As shown in Table 1, by 8 to 9 hr after exposure to progesterone, $^{45}Ca^{2+}$ uptake had decreased to about 20% of the control. In the experiment shown, nuclear breakdown began 10 to 11 hr after exposure to steroid, and by 12 to 13 hr $^{45}Ca^{2+}$ uptake in progesterone-treated oocytes had decreased to about 4% of the control. As with membrane potential (above), pretreatment with 1.8 μM cycloheximide completely blocked the progesterone-induced decrease in $^{45}Ca^{2+}$ uptake, whereas cycloheximide alone had no detectable effect on Ca^{2+} permeability.

[^{3}H]-Leucine and [^{32}P]-PO_4 Uptake and Exchange

In order to determine parameters for the uptake and exchange of leucine and PO_4, denuded oocytes were exposed to Ringer's solution containing 0.70 μM [^{3}H]-leucine or 80 μM [$^{32}PO_4$]. After 60 min a portion of the oocyte population was rinsed and transferred to Ringer's solution containing the same concentrations of unlabeled leucine or PO_4. As shown in Fig. 5, leucine uptake was essentially linear over the first 2 hr and no detectable leucine exchange with the medium was seen after 1 hr. By 2 hr the denuded oocyte had concentrated leucine six- to sevenfold from the medium into the acid-soluble pool. For comparison, PO_4 was not only concentrated 200-fold into the acid-soluble pool in 1 hr, but also exchanged with that in the medium. This efflux was essentially the same in PO_4-containing and PO_4-free medium (*data not shown*).

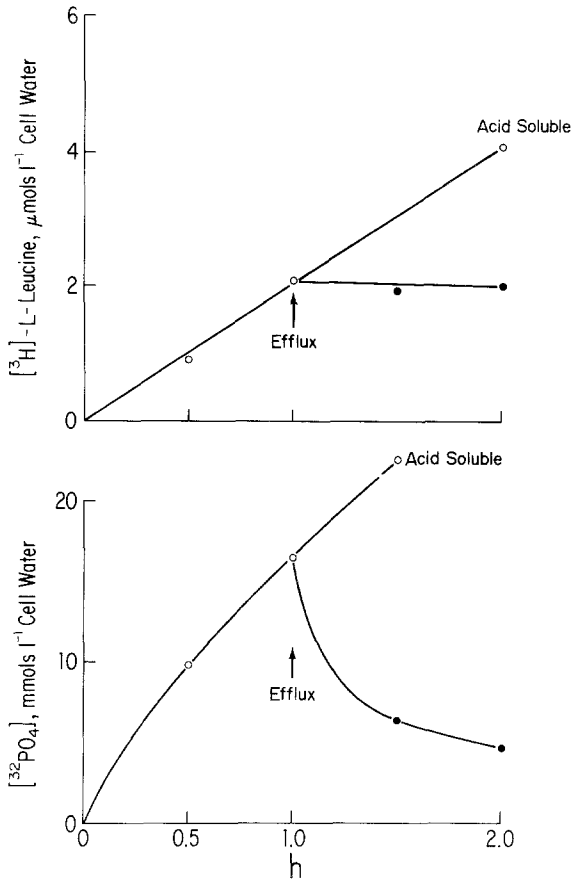

Fig. 5. Uptake of [^{3}H]-leucine (upper) and [$^{32}PO_4$] (lower) into the acid (TCA)-soluble phase by denuded *Rana* oocytes (no progesterone treatment). At the time point indicated by the arrow (efflux), a portion of the oocytes was transferred to ^{3}H-leucine-free and $^{32}PO_4$-free Ringer's solution and efflux into the medium was measured at the times indicated

Uptake of [^{3}H]-leucine and [^{32}P]-PO_4 into the acid-soluble pool was also monitored following continuous steroid treatment (1.6 μM progesterone) to determine if changes in uptake and/or acid-soluble pool size occurred during the depolarization phase of meiosis. As shown in Table 2, [^{3}H]-leucine and [^{32}P] uptake into the acid-soluble phase was relatively constant during successive 1-hr pulses, although the acid-soluble pool for both was larger in the unstimulated compared to the stimulated oocyte. If anything, there may be a small decrease in uptake following completion of nuclear breakdown. These results do not, however, take into consideration possible changes in intracellular free amino acids and/or inorganic phosphate or redistribution between internal pools (e.g. nucleus to cytoplasm). Although data are not shown, exposure to high [progesterone]$_o$ (3 to 32 μM markedly reduced [^{3}H]-leucine and/or [$^{32}PO_4$] uptake by denuded *Rana* oocytes within 1 hr after exposure.

Table 2. Uptake of [^{3}H]-leucine[a] and [^{32}P]-PO_4[b] into the acid-soluble pool during successive 60-min pulses during continuous exposure to progesterone[c]

Pulse period (hr)	% GVBD	[^{3}H]-leucine (μmols liter^{-1} h^{-1})		[^{32}P] (mmol liter^{-1} hr^{-1})	
		Untreated	Progesterone	Untreated	Progesterone
4–5	0	30.3	14.3	17.8	12.0
5–6	0	23.0	18.2	18.0	13.9
6–7	0	24.2	18.9	18.3	12.5
7–8	0	–	18.1	–	13.5
8–9	0	26.4	20.6	19.4	12.6
9–10	10	28.0	20.9	20.6	15.4
10–11	100	31.0	17.2	21.2	13.6
11–12	100	–	13.7	–	10.7
12–13	100	30.8	16.4	23.9	12.5
13–14	100	–	15.4	–	11.8

[a] $[Leucine]_o = 7.6$ μM. [b] $[Phosphate]_o = 80$ μM. [c] $[Progesterone]_o = 1.6$ μM.

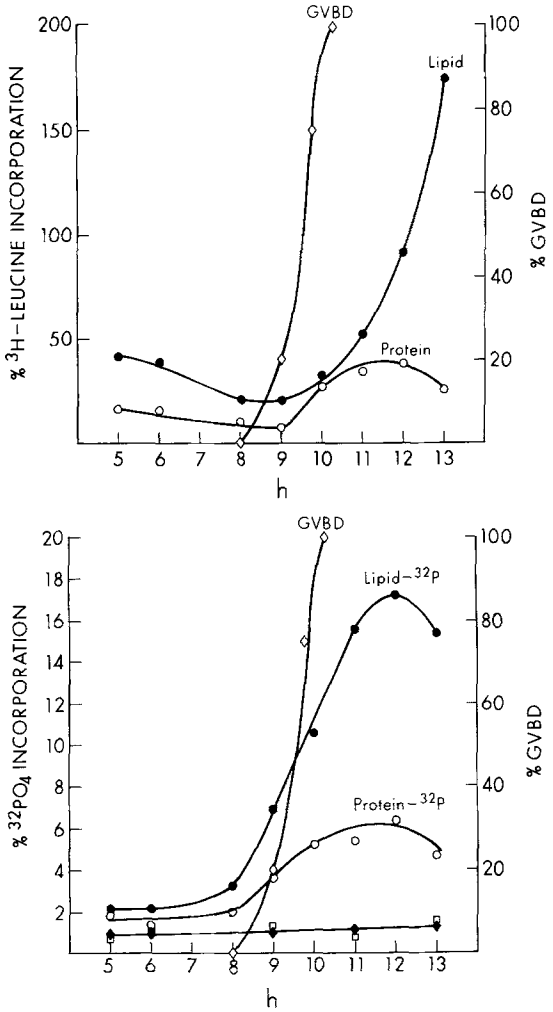

Fig. 6. Comparison of [^{3}H]-leucine (upper) and [$^{32}PO_4$] (lower) incorporation into protein (o—o) and lipid (●—●) fraction during successive 1-hr pulses in 1.6 μM progesterone-treated denuded oocytes. Uptake is expressed as % of the total acid-soluble pool. The time course of GVBD (◇—◇) is shown for comparison. □—□ and ◆—◆ in the lower graph indicate $^{32}[PO_4]$ incorporation into untreated (control) oocyte protein and lipid, respectively

Changes in [^{3}H]-Leucine Incorporation

[^{3}H]-leucine incorporation into both the protein and lipid phase in progesterone-induced denuded oocytes was measured during successive 1-hr pulses over the time course of nuclear breakdown and membrane depolarization. Oocytes were extracted successively with cold TCA, $CHCl_3/CH_3OH$ (2:1), and hot TCA to remove low molecular weight constituents, lipids and nucleic acids, respectively, as described in Materials and Methods. The final residue represents the protein and phosphoprotein fraction. The results shown in Fig. 6 are from a double-labeling experiment ([$^{32}PO_4$], [^{3}H]-leucine). In both Fig. 6 (upper) and Fig. 6 (lower), the rate of incorporation (% acid-soluble pool incorporated/hr) is compared with the time course of nuclear breakdown (% GVBD). As illustrated in Fig. 6 upper, [^{3}H] leucine was incorporated into both protein and lipid. It was incorporated into protein most rapidly 1 to 2 hr following maximal rate of nuclear breakdown. Its maximum rate of incorporation into the lipid phase, however, occurred at least 2 to 3 hr after the maximum rate of protein synthesis. For comparison, [^{3}H]-leucine incorporation by untreated, denuded oocytes was relatively constant with 6.4 and 5.0% incorporation per hour into lipid phase and protein, respectively. As shown in Table 2 (above), there was no apparent change in uptake or acid-soluble pool size over the first 13 to 14 hr after exposure to progesterone.

The [^{3}H]-leucine recovered in the $CHCl_3/CH_3OH$ (lipid) extract could not be removed by standard techniques designed to remove small molecular weight contaminants from crude lipid extracts [3]. When the $CHCl_3/CH_3OH$ extract was taken to dryness and re-extracted with $CHCl_3/CH_3OH$ (2:1), a residue remained that was only

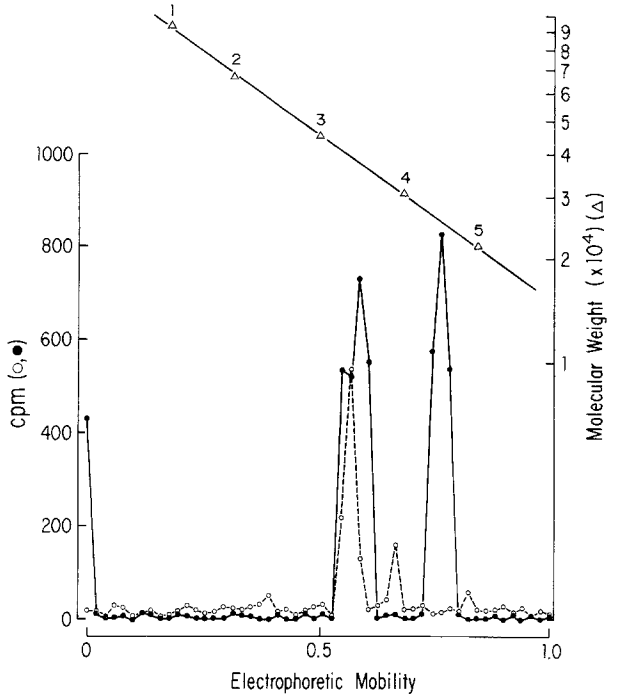

Fig. 7. Comparison of the electrophoretic patterns of [^{32}P]-phosphorylated proteins from the membrane complex (•—•) with [^{3}H]-leucine incorporation into the proteolipids of the whole oocyte (o—o). Material shown was pooled from experiments outlined in Tables 2 and 3, respectively. Marker proteins were phosphorylase B (1), BSA (2), ovalbumin (3), carbonic anhydrase (4), and soybean trypsin inhibitor (5)

partly soluble in $CHCl_3/CH_3OH$ or in water and contained most of the [^{3}H] taken up into the lipid phase. Electrophoresis of pooled material from 9 to 12 hr on SDS-polyacrylamide gels demonstrated a number of protein bands, two of which contained significant [^{3}H]-label (*see* Fig. 7). Similar lipoprotein material was subsequently isolated using the $CHCl_3/CH_3OH/KCl$ extraction method of Folch-Pi and Stoffyn [4]. No significant [^{3}H] co-migrated with specific phospholipids on thin-layer chromatography.

[^{3}H]-leucine incorporation into total protein, lipoprotein, and plasma-vitelline membrane is compared in Table 3. As shown, there was a threefold increase in [^{3}H]-leucine incorporation into protein when compared to uninduced prophase oocytes. This [^{3}H]-leucine incorporation was largely associated with the lipoprotein and plasma-vitelline membrane complex. A time course of [^{3}H]-leucine uptake into the plasma-vitelline membrane, TCA-insoluble, and TCA-soluble fractions appears elsewhere [37]. Progesterone-stimulated [^{3}H]-leucine and [$^{32}PO_4$] uptake into the plasma-vitelline membrane fraction was essentially the same in intact and manually enucleated [41] oocytes.

Table 3. [^{3}H]-leucine incorporation into macromolecular fractions during the time-course of nuclear breakdown in *R. pipiens* oocytes

Fraction	[^{3}H]-leucine incorporation, dpm/oocyte[a]	
	Control	Progesterone-induced
Protein (Total)	1,300	4,127
Proteolipid[b]	602	3,328
Plasma-vitelline membrane	423	2,731

[a] Six-hr pulse beginning with onset of nuclear breakdown (GVBD). GVBD occurred 9 to 11 hr after initial exposure to 3.2 μM progesterone.
[b] Method of Folch-Pi and Stoffyn, 1972 [3].

Changes in [^{32}P]-PO_4 Incorporation

An increased rate of incorporation of [$^{32}PO_3$] into both bulk phosphoprotein and into the lipid-soluble components was found during successive 1-hr pulses beginning at about the onset of nuclear breakdown (8 to 9 hr), reaching a peak about 2 hr after completion of nuclear breakdown (Fig. 6, lower). In the study shown, [$^{32}PO_4$] incorporation into lipid-associated components was about 3× that of the bulk protein. Analysis of lipid-soluble material indicated that about 25 to 30% of the [$^{32}PO_4$] was recovered as phospholipid. Of the remaining 70 to 75% of the [^{32}P], nearly all became insoluble in $CHCl_3/CH_3OH$ (2:1) after taking the original extract to dryness and were shown to be largely associated with proteolipids when analyzed by PAGE.

In the experiments measuring $^{32}PO_4$ incorporation (Fig. 6, lower), nuclear breakdown preceded maximal phosphorylation of components in both bulk protein and lipid phase. Analysis of data from three such experiments indicates that nuclear breakdown precedes maximal phosphorylation by 1 to 4 hr. As shown in Table 2, uptake of [$^{32}PO_4$] into the acid-soluble pool during successive 1-hr pulses was relatively constant in both progesterone-induced and uninduced oocytes although, as with [^{3}H]-leucine, uptake was somewhat greater in the acid-soluble pool in the uninduced oocytes. When oocytes were pulsed for 1 hr during nuclear breakdown and then transferred to [$^{31}PO_4$] containing Ringer's solution, 40% of the acid soluble [$^{32}PO_4$] exchanges with that of the medium within 1 hr and about 70% by the end of 3 hr. For comparison, 70% of the acid-soluble [$^{32}PO_4$] in prophase oocytes exchanges with that of the medium within 1 hr (Fig. 5).

To determine the cellular localization of [^{32}P]-labeled protein and phospholipid, oocytes were

pulse labeled with [$^{32}PO_4$] for 4 hr beginning with the onset of nuclear breakdown. At the end of the pulse, the plasma-vitelline membrane complex was isolated (*see* Materials and Methods) and both the membrane complex and cytoplasm were extracted successively with 6% TCA (4 °C) and $CHCl_3/CH_3OH$ (2:1). The distribution of [^{32}P] between protein and phospholipid in both membranes and cytoplasm is compared for control and progesterone-treated oocytes in Table 4. Lipid-[^{32}P] represents about 40% of the [^{32}P] incorporated into the membrane complex of control oocytes with no significant [^{32}P] uptake into cytoplasmic lipids. In progesterone-treated oocytes there was a 14-fold increase in membrane-[^{32}P], with about 25% associated with membrane lipids. [^{32}P] incorporation increased more than 16-fold into membrane proteins but only about ninefold into membrane lipids. [^{32}P] incorporation into cytoplasmic proteins also increased (10-fold) and accounted for about 60% of the increase in total protein phosphorylation in the oocyte undergoing and following nuclear breakdown. During this time, there was a 30% decrease in the acid-soluble [^{32}P] pool in progesterone-treated oocytes.

The electrophoretic pattern of [^{32}P]-labeled protein extracted from the plasma-vitelline membrane complex is compared with [^3H]-leucine incorporation into the lipoprotein fraction in post-GVBD oocytes (in Fig. 7). Major phosphorylated peaks were found in the 20,000 and 40,000 mol wt regions with a small amount of higher molecular weight material at the origin. The major [^3H]-leucine-labeled peak co-electrophoresed with the 40,000 mol wt phosphorylated membrane protein(s) with a smaller [^3H]-labeled component migrating between the two phosphorylated membrane components. Further studies are in progress to compare the pattern of newly synthesized and/or phosphorylated proteins from plasma membranes, lipoproteins and oocyte cytoplasm by two-dimensional gel electrophoresis.

The crude lipids extracted from the oocytes in these experiments were analyzed on two-dimensional thin-layer chromatography and six phosphatides were found to constitute 90% of the total phospholipid phosphorus. Phosphatidylcholine and phosphatidylethanolamine were the major phosphatides with smaller amounts of phosphatidylserine, lysophosphatidylcholine, phosphatidic acid, and phosphatidylinositol. The percent distribution of the various phospholipid components in prophase oocytes is presented in Table 5. There was no statistical difference in the total phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine content of oocytes prior to and following nuclear breakdown. Table 5 includes a comparison of the specific acitivity of the individual phosphatides following nuclear breakdown with prophase oocytes from the same female incubated for the same time period without progesterone. Oocytes from four successive 1-hr pulse-label-

Table 4. Comparison of [$^{32}PO_4$] incorporation into plasma membrane and cytoplasmic proteins and phospholipids: Effect of progesterone

Treatment	[^{32}P] cpm/oocyte[a]				
	Plasma membrane		Cytoplasm		
	protein	lipid	soluble	protein	lipid
Control	680[b] ±110	420 ±60	87,400 ±11,400	1,580 ±170	< 40
Progesterone	11,200 ±1,750	3,660 ±1,420	57,800 ±5,400	16,200 ±1,830	<260

[a] Measured 11 to 14 hr after exposure to progesterone.
[b] Mean ± SEM ($n=4$).

Table 5. Phospholipid phosphorus turnover during the time-course of nuclear breakdown in *R. pipiens* oocytes[a]

Phospholipid	% Total phospholipid-P	^{32}P Incorporation, cpm/μmol		% Change in turnover
		Control	Progesterone-induced	
Phosphatidylcholine	56.3	881	303	34
Lysophosphatidylcholine	1.3	2,600	7,040	270
Phosphatidylethanolamine	26.6	2,250	3,490	303
Phosphatidylserine	4.4	2,390	4,720	340
Phosphatidylinositol	0.73	2,370	2,130	90
Phosphatidic acid	4.6	1,000	3,700	370

[a] $CHCl_3/CH_3OH/KCl$ extracts of sibling oocytes from 4 successive 1-hr pulses following nuclear breakdown (*see* Materials and Methods).

ing periods following nuclear breakdown were pooled and extracted with $CHCl_3/CH_3OH/KCl$ as described under Material and Methods. Lysophosphatidylcholine and phosphatidylinositol appeared to turn over more than twice as rapidly as serine, ethanolamine or choline containing phosphatides in control oocytes. In contrast, following nuclear breakdown there was a marked increase in specific activity of lysophosphatidylcholine and serine and ethanolamine-containing phosphatides and a significant decrease in specific activity of phosphatidylcholine. Phosphate uptake into phosphatidylinositide remained relatively unchanged. The largest increase in uptake was seen in the phosphatidic acid fraction.

Discussion

The results presented here indicate that a marked increase in protein synthesis, protein phosphorylation, and phosphorylation of specific lipids occurs in the oocyte plasma membrane during the depolarization phase which terminates with the appearance of an excitable membrane late in the first meiotic division. Previous studies in this laboratory had shown [41] that progesterone-induced plasma membrane depolarization began at about the time of nuclear breakdown. Using more precise measurements, we report here that the oocyte first depolarizes from about -60 mV (inside negative) to about -25 mV 1 to 2 hr before breakdown of the large nucleus. This observation is particularly interesting in that it suggests that depolarization might serve as a trigger for nuclear breakdown. However, we have previously shown that preventing plasma membrane depolarization using a voltage-clamp does not prevent nuclear breakdown [38]. The first depolarization is followed by a slow depolarization phase beginning 3 to 6 hr after nuclear breakdown. The depolarization prior to nuclear breakdown coincides with the disappearance of both K^+ permeability [21] and an electrogenic Na^+, K^+-pump [37, 41]. Disappearance of the Cl^- conductance [39] appears to occur during the second depolarization phase.

As shown here and elsewhere [37], there was no detectable change in [^{3}H]-leucine incorporation into membrane and/or cytoplasmic proteins prior to or during the depolarization phase that precedes nuclear breakdown. Masui and coworkers have shown [12, 35] that nuclear breakdown is dependent upon production of a cytoplasmic factor (called "maturation promoting factor" or MPF) and that production of the factor occurs in the absence of the nucleus and is blocked by cycloheximide. Similarly, we have previously shown [41] that progesterone-induced depolarization occurs in enucleated oocytes and is inhibited by cycloheximide. As reported here, cycloheximide concentrations have the same ED_{50} for inhibition of progesterone-induced nuclear breakdown, plasma membrane depolarization, and [^{3}H]-leucine incorporation. As also shown, the period of cycloheximide sensitivity (first 5 hr) is essentially the same both for the first phase of plasma membrane depolarization and for nuclear breakdown. As reported elsewhere [26], the continuous presence of progesterone is required during this initial 5-hr period. These results are consistent with a model in which "MPF" is continuously synthesized and progesterone inhibits "MPF" degradation. The "MPF" in turn may act to specifically down-regulate or internalize the plasma membrane Na^+,K^+-ATPase [37] as well as K^+ channels [21, 41], producing the first depolarization phase.

In contrast, the onset of the second depolarization phase is preceded by an increase in rate of [^{3}H]-leucine and [^{32}P] incorporation into both protein and lipid. The most marked increase in [^{3}H]-leucine incorporation is into a proteolipid fraction; incorporation continues to rise during the second depolarization phase. This proteolipid appears associated with the oocyte plasma membrane (Table 2 and Fig. 7). Folch-Pi and Stoffyn [4] have described proteolipids with similar solubility characteristics in brain, and proteolipids are also found in a wide variety of animal and plant tissues [4].

As shown here, oocytes rapidly concentrate amino acids and inorganic phosphate from the medium. A major problem in measuring incorporation rates lies in assessing precursor pool sizes. Previous studies have reported [13, 23] that amino acids and inorganic PO_4 taken up from the medium are largely accumulated in the oocyte nucleus. In contrast, amino acids microinjected into the oocyte are reported to diffuse throughout the oocyte [27]. Thus, incorporation patterns may differ depending on the route of administration. Ecker [2] has suggested that most of the so-called free amino acids in the oocyte are not available for protein synthesis and that earlier kinetic studies using injected amino acids were incorrect. More recently Wasserman et al. [34, 36] report that either progesterone or injected MPF produce a twofold increase in the absolute rate of protein synthesis in *Xenopus* oocytes. These workers report "minimal changes in the types of polypeptides" in oocytes injected not less than 60 min after nuclear breakdown. In contrast, YoungLai et al. [40] report at least five polypeptide "modifications" during progesterone-

induced meiosis in the same species when labeled amino acids are added to the medium.

In the present study, [^{3}H]-leucine uptake into the total acid-soluble pool was relatively constant during the first meiotic division and an apparent increase in incorporation occurred following nuclear breakdown. This was seen as increased rates of total incorporation, membrane incorporation, and incorporation into proteins analyzed by PAGE. A large fraction of the incorporated [^{3}H]-leucine was recovered in the lipid phase and was associated with two protein peaks on PAGE. This proteolipid would have been discarded in the studies by Smith and Ecker [29], Shih et al. [27] or Wasserman et al. [36]. Masui and Clarke [11] have stated (but not presented data) that "free leucine in TCA precipitates is difficult to remove, while the method using perchloric acid (PCA) instead of TCA gives more accurate results." We find that less than 1% of the [^{3}H]-leucine uptake into TCA precipitable protein is due to nonspecific binding. As shown in Fig. 2, cycloheximide inhibits [^{3}H]-leucine incorporation by 90 to 95% indicating incorporation, not nonspecific binding. The use of PCA at 60 °C may extract proteolipid and might have been interpreted as "free" leucine.

Vilain et al. [32] have reported that 18 μM cycloheximide prevents a progesterone-induced surge in "free" cytosolic Ca^{2+} that occurs shortly before nuclear breakdown in the *Ambystoma* oocyte. These authors suggest that the inhibitory effect of cycloheximide is on Ca^{2+} "release" within the oocyte and is unrelated to its effect on protein synthesis. They also state (but do not present data) that the protein synthesis inhibitor emetine "did not block maturation at those concentrations which abolished protein synthesis" in *Marthasterias glacialis* oocytes. We report here that emetine (at concentrations from 1 nM to 1 mM) inhibits neither [^{3}H]-leucine incorporation nor progesterone-induced membrane depolarization and nuclear breakdown in *Rana* oocytes. Emetine is an effective protein synthesis inhibitor in cell-free systems and mammalian cells in culture [24] but does not readily enter all eukaryotic cells (Dr. Jonathan Warner, *personal communication*). We find that cycloheximide also inhibited the progesterone-induced decrease in Ca^{2+} permeability that occurs during the first depolarization phase in *Rana* oocytes (Table 1). This decrease in Ca^{2+} permeability appears to coincide with the release of cytosolic Ca^{2+} seen by Vilain et al. [32]. The increase in free $[Ca^{2+}]_i$ may be causally related to the decrease in Ca^{2+} permeability in a manner analogous to that proposed for inactivation of voltage-dependent Ca^{2+} channels (Ca^{2+}-induced Ca^{2+} channel inactivation, reviewed in ref. 31). Our earlier finding that exogenous Ca^{2+} is required for inhibition of nuclear breakdown by DBcAMP or theophylline but not cycloheximide [18] indicates that there is a Ca^{2+}-dependent event and a protein synthesis-dependent event which precede nuclear breakdown. The inhibition of both the decrease in Ca^{2+} permeability (Table 1) and "release" of cytosolic Ca^{2+} [32] by cycloheximide indicates that a progesterone-induced protein synthetic step is essential to both.

The increase in protein phosphorylation seen here is in good agreement with studies by Maller et al. [10] in which [$^{32}PO_4$] was microinjected into *Xenopus* oocytes with an intact follicle epithelium. In both *Rana* and *Xenopus*, protein phosphorylation increased at about the time of nuclear breakdown. A major difference is that we find a significant increase in phospholipid phosphorylation following nuclear breadkown whereas Maller et al. [10] report only 5.4% of the acid-insoluble [^{32}P] is recovered as phospholipid ($CHCl_3/CH_3OH$ extracted) in progesterone-treated follicles. We find that *Rana* oocytes concentrate PO_4 from the medium and that PO_4 taken up by the denuded oocyte (or follicle) rapidly exchanges with that in the medium. Since the studies by Maller et al. [10] were carried out in PO_4-free medium, $[PO_4]_i$ may have become rate limiting. Thus, incorporation may be different with injected [$^{32}PO_4$] and/or [$^{32}PO_4$] incorporation into phospholipid may have been depressed in the *Xenopus* studies. We have previously reported [15] that a PO_4-containing medium is essential for a synchronous time course of meiosis in *Rana* oocyte populations.

In summary, the present study indicates that in *Rana* oocytes nearly all of the increase in leucine incorporation and about one-half the increase in protein phosphorylation during the first meiotic division is associated with the plasma-vitelline membrane complex. These plasma membrane changes appear to be independent of nuclear breakdown but may be associated with the slow depolarization phase late in the first meiotic division. An increase in phosphate incorporation into phosphatidylserine and phosphatidylethanolamine with a concomittant decrease into phosphatidylcholine also begins during the slow depolarization phase. Phosphatidylserine and phosphatidylethanolamine appear to be located predominantly on the cytoplasmic side of the plasma membrane in other cells whereas phosphatidylcholine is mainly outside [25]. Thus, a marked increase in both membrane asymmetry (amine phospholipids) as well as protein negative charge (phosphorylation) appear

to be taking place as Cl^- conductance disappears [39] coincident with the appearance of an excitable egg membrane late in the first meiotic division. We had previously shown [17] that the phosphate incorporated into egg proteins during the meiotic divisions is released during increased Cl^- conductance [16] within the first few minutes following fertilization. Studies are now in progress to correlate changes in membrane conductance to specific ions (e.g., Cl^-) and membrane excitability with both phosphorylation and dephosphorylation of specific membrane proteins, phosphatidopeptides and phospholipids.

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