J. Membrane Biol. 69, 41–48 (1982)

The Journal of
Membrane Biology

Progesterone-Induced Down-Regulation of an Electrogenic Na^+, K^+-ATPase during the First Meiotic Division in Amphibian Oocytes

Steven P. Weinstein, Adele B. Kostellow, David H. Ziegler, and Gene A. Morrill
Department of Physiology and Biophysics, Albert Einstein College of Medicine, New York, New York 10461

Summary. Progesterone initiates the resumption of the meiotic divisions in the amphibian oocyte. Depolarization of the *Rana pipiens* oocyte plasma membrane begins 6–10 hr after exposure to progesterone (1–2 hr before nuclear breakdown). The oocyte cytoplasm becomes essentially isopotential with the medium by the end of the first meiotic division (20–22 hr). Voltage-clamp studies indicate that the depolarization coincides with the disappearance of an electrogenic Na^+, K^+-pump, and other electrophysiological studies indicate a decrease in both K^+ and Cl^- conductances of the oocyte plasma membrane. Measurement of [^{3}H]-ouabain binding to the plasma-vitelline membrane complex indicates that there are high-affinity ($K_d = 4.2 \times 10^{-8}$ M), K^+-sensitive ouabain-binding sites on the unstimulated (prophase-arrest) oocyte and that ouabain binding virtually disappears during membrane depolarization. [^{3}H]-Leucine incorporation into the plasma-vitelline membrane complex increased ninefold during depolarization with no significant change in uptake or incorporation into cytoplasmic proteins or acid soluble pool(s). This together with previous findings suggests that progesterone acts at a translational level to produce a cytoplasmic factor(s) that down-regulates the membrane Na^+, K^+-ATPase and alters the ion permeability and transport properties of both nuclear and plasma membranes.

Key words progesterone · oocyte · Na^+, K^+-ATPase · ouabain · electrogenic

Introduction

The vertebrate oocyte is arrested in first meiotic prophase until hormonal release at the time of ovulation. The oocyte then completes one and one-half meiotic divisions and is again arrested at second meiotic metaphase. Fertilization allows completion of the meiotic divisions and is followed by the cleavage phase of development. During the meiotic divisions, the oocyte plasma membrane undergoes a depolarization near the end of the first meiotic division and repolarizes during the first mitotic division after fertilization [2, 14, 18, 24, 27]. Studies with oocytes of *Rana* indicate [27] that the onset of depolarization coincides with the swelling and subsequent breakdown of the large nucleus (some 8–12 hr after hormonal reiniation of the first meiotic division), the oocyte cytoplasm becoming essentially isopotential with the medium by the completion of the first meiotic division (20–22 hr). This depolarization is associated, in part, with a decrease in K^+ permeability of the plasma membrane [19, 27] and is a prelude to the development of an excitable state early in the second meiotic division [1].

Previous electrophysiological studies in this laboratory [27] and by Wallace and Steinhardt [24] have suggested that a substantial portion (up to 40 mV) of the resting potential of denuded, prophase-arrested oocytes is due to an electrogenic Na^+, K^+-pump. Biochemical studies [15, 16] have shown that the ouabain-sensitive Na^+, K^+-ATPase essentially disappears during the depolarization phase of meiosis, suggesting that one site of hormone action may be the membrane Na^+, K^+-pump. The present study was undertaken in order to characterize the properties of the oocyte membrane Na^+, K^+-ATPase at different times during the first meiotic division. The biochemical and biophysical characterization of the plasma membrane Na^+, K^+-ATPase in *Rana* oocytes is greatly facilitated by the unusually large size of the cell (1,800 μm diameter) and by the relative ease of manual removal of the intact plasma membrane. As outlined below, the integrity of the vitelline envelope (a meshwork of fibers closely applied to the oocyte surface [31]) makes it possible to isolate the intact plasma membrane. The relatively long time course (minutes to hours) of depolarization-repolarization [15, 18] in oocytes during ovulation and fertilization (the meiotic divisions) makes it possible to study the biochemical events underlying the electrical events in an excitable cell. A preliminary report has been presented [26].

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

0022-2631/82/0069-0041 $01.60

cells were prepared by the method of Masui [11] with the modifications described elsewhere [27]. The Ringer's solution used throughout these studies is a modified amphibian Ringer's solution containing 111 mM NaCl, 1.9 mM KCl, 1.1 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 0.08 mM NaH_2PO_4, and 0.8 mM $MgSO_4$.

Progesterone, 17β-estradiol, and strophanthidin (Sigma Chemical Co., St. Louis, Mo.) were prepared in 95% ethanol, and 1 µl/ml was added per ml of Ringer's solution, whereas ouabain (Sigma Chemical Co., St. Louis Mo.) was dissolved directly in Ringers. [$\gamma-{}^{32}$P]-ATP, tetra (triethylammonium) salt (sp act, 3000 Ci/mmol), [^{3}H(G)]-ouabain (22.6 Ci/mmol), [^{3}H]-L-leucine (110–145 Ci/mmol), ^{42}KCl, and ^{22}NaCl were obtained from New England Nuclear (Boston, Mass.).

Meiotic Agonists: Nuclear Breakdown

Twenty denuded oocytes were incubated at 20–22 °C in 10 ml Ringer's solution containing agonists as indicated. Untreated controls included oocytes in Ringer's solution as well as Ringer's solution with 1 µl/ml ethanol added. The various inducing agents were 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.

[^{3}H]-Ouabain and $^{42}K^+$ Uptake and Exchange, and $^{22}Na^+$ Efflux

Thirty denuded oocytes per experiment were incubated in 4.0 ml Ringer's solution containing [^{3}H]-labeled ouabain as outlined in the individual experiments. In some experiments, $[K^+]_o$ was increased by isomolar substitution of KCl for some or all of the NaCl in the standard Ringer's. Oocytes were kept slowly moving and were handled using glass pipettes with a bore diameter slightly larger than the oocyte. At 5, 15, 30, and 60 min, six oocytes were removed, rinsed five times with 50 ml aliquots of Ringer's solution, and transferred to a buffered sucrose solution as described below. Uptake was measured at 20–22 °C. The volume of oocytes added was less than 2% of the volume of the incubation medium, and in no case did oocytes take up more than 1% of the added $^{42}K^+$ or [^{3}H] after 60 min.

$^{42}K^+$ uptake and exchange and $^{22}Na^+$ efflux from denuded oocytes have been described previously [19].

Isolation of Nuclei and Plasma-Vitelline Membrane Complex

Six oocytes were 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 20–22 °C. A small slit was made in the animal hemisphere, and the nucleus was expressed through the slit by slight pressure on the oocyte with fine-tipped forceps. The nuclei were quickly collected with a Pasteur pipette, rinsed with two 3.0-ml volumes of buffered sucrose solution (above), and transferred to a scintillation vial for counting. The enucleated oocytes were then opened further with forceps and the plasma-vitelline membrane complex everted and drawn into a Pasteur pipette. Movement of the membrane complex up and down within the pipette resulted in a rapid displacement of residual platelets and granules; the clear membrane complex was then passed through two 3.0-ml volumes of buffered sucrose solution and counted. Total time for recovery of nuclei and membranes from six oocytes was about 20 min.

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. Oocytes were voltage-clamped by feeding the membrane potential signal into a negative feedback circuit, the ouput of which was fed back into the oocyte via a second micropipette. Clamping current was measured by recording the voltage drop across a resistor placed in series with the feedback micropipette. In a typical experiment, an oocyte was clamped at its original resting potential. A series of voltage steps was then imposed upon the oocyte; the "instantaneous" clamping-currents were measured, and the oocyte membrane conductance was measured as the slope of the line $I=gE$ as determined by linear regression analysis. When strophanthidin was added, the oocyte membrane current was allowed to approach a new steady-state and the series of voltage steps was repeated. All studies were carried out at 20–22 °C using modified Ringer's solution (*see* above), Li^+-Ringer's solution (LiCl substituted for NaCl), or 5 × $[K^+]_o$ Ringers (i.e., Ringers in which $[K^+]_o$ was increased to 9.5 mM by the addition of KCl).

ATPase Activity Measurements

Denuded oocytes were homogenized (150 mg wet wt ml^{-1}) in distilled water and 0.2 ml added to 2.3 ml medium containing a final concentration of 0.5 mM ATP, 5.0 mM HEPES buffer (pH 7.4), 10 mM NaCl, 100 mM KCl, 2.5 mM $MgCl_2$, and 500,000 dpm (γ-^{32}P]-ATP, Tetra(triethylammonium) salt (sp act, 3000 Ci/mmol). The reaction mixture was incubated at 26 °C, and 0.4 ml aliquots were removed at 0, 5, 15, 30 and 60 min. Each aliquot was treated immediately with 0.4 ml 10% TCA at 0–4 °C, centrifuged at 3,000 × g for 15 min, and the $^{32}P_i$ extracted from the supernatant by the method of Petzelt [21]. Basal ATPase levels were measured in medium containing 110 mM NaCl.

[^{3}H]-Leucine Incorporation

Denuded oocytes were incubated at 20–22 °C in Ringer's solution (5 oocytes/ml) containing 0.09 µM [^{3}H]-L-leucine (sp act 57–130 Ci/mmol) and 0 or 1.65 µM progesterone. Care was taken to insure that the $[leucine]_o$ did not become rate-limiting; in the experiments shown, less than 5% of the leucine in the medium was taken up by the oocytes. At the times indicated, 5 to 10 oocytes were removed from the incubation medium, rinsed five times with Ringer's solution, and the plasma-vitelline membrane complex was isolated as described above. Membranes were either counted directly or were treated on Whatman GF/C filters with 2.0 ml 6% TCA at 4 °C for 15 min, rinsed, and then counted. A 0.5-ml aliquot of cytoplasm was counted directly, as well as after TCA-precipitation (6% TCA, final concentration, at 4 °C for 15 min) and collection on a GF/C filter. [^{3}H] was measured using an Intertechniques Scintillation Spectrometer, and counts were corrected for quenching using an external standard.

Results

Changes in Plasma Membrane Properties and Na^+, K^+-ATPase Activity during the First Meiotic Division

The electrophysiological properties of voltage-clamped *Rana* oocytes arrested at first meiotic prophase are compared with those of oocytes completing the first meiotic division (20–25 hr) in Table 1. Treatment of voltage-clamped, prophase oocytes with 10^{-5} M strophanthidin resulted in a rapid (< 1 min) decrease in membrane current without a significant change in membrane conductance, whereas treatment

Table 1. The effect of strophanthidin on the electrical properties of denuded *Rana* oocytes during the first meiotic division[a]

Preparation	$E_{I=0}$ (mV)	I (nA)	g (μs)
Prophase-arrest (18,9)[b]	-59 ± 2.8	–	4.4 ± 0.5
Prophase-arrest + 10^{-5} M strophanthidin (18,9)	-42 ± 2.9	$-63\ \pm8$	4.1 ± 0.5
Post-GVBD,[c] 17–22 hr (15,3)	-18 ± 1.9	–	2.0 ± 0.4
Post-GVBD + 10^{-5} M (15,3) strophanthidin	-17 ± 1.2	$-\ 1.6\pm1.4$	1.6 ± 0.3

[a] $E_{I=0}$ is the potential at which the membrane current is equal to zero. This is equal to the resting potential in the case of untreated oocytes, and is usually no more than a few millivolts different from the resting potential in treated eggs, the difference being due to small voltage-dependent conductance changes. I is the current necessary to maintain the oocyte at its original resting potential. g is the "instantaneous" oocyte conductance.
[b] Values are mean ± SEM (No. of oocytes, No. of frogs).
[c] Germinal vesicle (nuclear) breakdown.

Table 2. The effect of Li^+-Ringer's solution on the electrical properties[a] of denuded prophase-arrest *Rana* oocytes

Preparation	$E_{I=0}$ (mV)	I (nA)	g (μs)
Prophase-arrest (10,1)[b]	-79 ± 1.3	–	–
Prophase-arrest (10,2) in Li^+-Ringer's solution[c]	-49 ± 1.2	–	3.6 ± 0.4^P
Prophase-arrest (3,1) in Li^+-Ringer's solution + 10^{-5} M strophanthidin[d]	-44 ± 2.4	-7 ± 1.7	3.7 ± 0.3

[a] $E_{I=0}$, I, and g are defined in Table 1.
[b] Values are the mean ± SEM (No. of oocytes, No. frogs).
[c] Oocytes were denuded and maintained in Li^+-Ringer's solution. The total time in Li^+-Ringer's solution was 2 to 3 hr.
[d] Three oocytes were voltage-clamped at the resting potential and the oocyte current and conductance was measured (*see* Methods) before and after the addition of 10^{-5} M strophanthidin.

of oocytes completing the first meiotic division with 10^{-5} M strophanthidin resulted in neither a change in membrane current nor a change in conductance.

Four observations provide additional evidence that the effect of strophanthidin on plasma membrane current is via inhibition of an electrogenic Na^+, K^+-ATPase. First, since intracellular Li^+ is transported by the Na^+, K^+-pump at a much slower rate than is Na^+ [8], incubation in Li^+-Ringer's solution would be expected to decrease or abolish an electrogenic resting potential component. As shown in Table 2, prophase oocytes which had been maintained in Li^+-Ringer's solution had resting potentials which were much lower (depolarized) than those of control oocytes. Addition of 10^{-5} M strophanthidin to Li^+-treated oocytes resulted in minimal further changes in resting potential, membrane current, and membrane conductance. Second, since it can be argued that a strophanthidin-induced decrease in membrane current may be due to extracellular K^+ accumulation, it is necessary to show that a current change of at least equal magnitude is observed in higher $[K^+]_o$

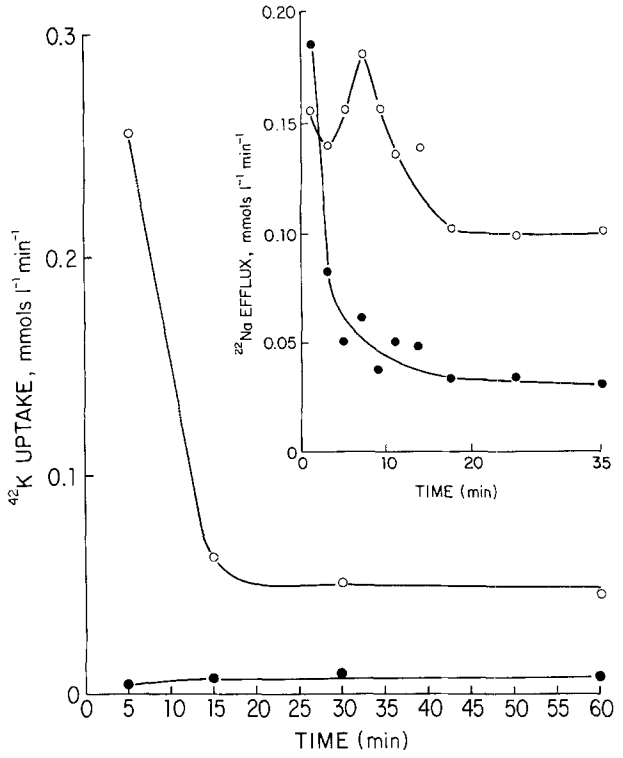

Fig. 1. The time course of $^{42}K^+$ uptake and $^{22}Na^+$ efflux (*inset*) in untreated (○—○) and strophanthidin (10^{-5} M)-treated (●—●) prophase oocytes. Oocytes were either (i) pretreated for 2 min with Ringer's solution containing zero or 10^{-5} M strophanthidin prior to beginning $^{42}K^+$ uptake in similar media, or (ii) preloaded with $^{22}Na^+$ for 2 hr and then rinsed with Ringer's solution for 1 min prior to transfer to Ringer's solution containing either zero or 10^{-5} M strophanthidin. Estimates of the Na^+, K^+-pump coupling ratio and the electrogenic current can be derived from the measured steady-state fluxes by assuming: (i) that the resting potentials of untreated and strophanthidin-treated oocytes were about -60 and -40 mV, respectively (Table 1), (ii) that the strophanthidin-induced depolarization does not significantly affect plasma membrane ion permeability, and (iii) that fluxes measured in the presence of strophanthidin reflect passive ion movements. Thus, the steady-state fluxes measured in strophanthidin-treated oocytes at 30 and 60 min (60 min points not shown for $^{22}Na^+$) were corrected to a resting potential of -60 mV by multiplying the fluxes by the ratio a/b, where a and b are equal to $(VF/RT)/(1\text{-exp}\ (-VF/RT))$ at -60 and -40 mV, respectively, and where V is the membrane potential (whose sign is positive if it assists ion movement). R, T, and F have their usual meanings. Subtraction of the corrected fluxes from those measured in untreated oocytes results in estimates of fluxes attributable to the Na^+, K^+-pump: 0.077 and 0.061 mmol Na^+ liter^{-1} min^{-1} and 0.037 and 0.035 mmol K^+ liter^{-1} min^{-1} at 30 and 60 min, respectively. The Na^+, K^+-pump coupling ratio is thus estimated to be between 3 Na^+:2K^+ and 2 Na^+:1K^+, and the electrogenic current predicted from these data is between 65 and 100 nA per oocyte.

Table 3. Comparison of strophanthidin-sensitive Na^+, K^+-ATPase activity[a] in prophase arrested and post-GVBD stage *Rana* oocytes

Preparation	P_i released pmol mg^{-1} min^{-1}	
	Homogenate	Homogenate + 1 mM strophanthidin
Prophase arrest	483 ± 64 (4)[b]	154 ± 24 (4)
Post-GVBD[c]	86 ± 105 (3)	602 ± 60 (3)

[a] Maximal strophanthidin-sensitive ATPase activity was obtained with incubation medium containing 100 mM K^+ and 10 mM Na^+. In these studies the rate of P_i released over the first 15 min was corrected for ATPase activity in K^+-free medium.
[b] Mean ± SEM (No. of experiments).
[c] 17–20 hr after initial exposure to 3.2 μM progesterone.

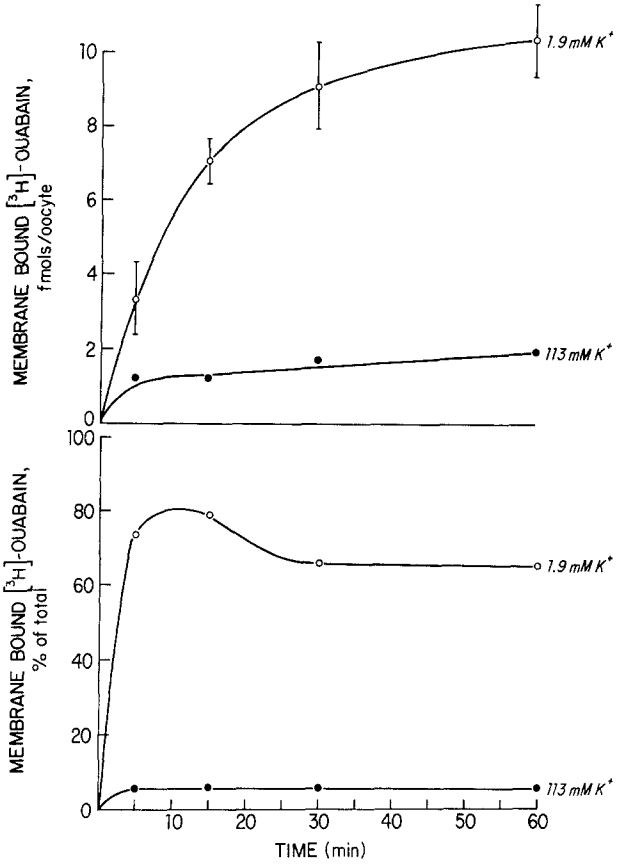

Fig. 2. *Upper:* Uptake of [^{3}H]-ouabain (9×10^{-8} M, final sp act 12 Ci/mmol) by the plasma-vitelline membrane complex in physiological $[K^+]_o$ (1.9 mM o—o) and high $[K^+]_o$ (113 mM, ●—●) Ringers' solution. Uptake is expressed as fmol/oocyte. (Bars indicate SEM for five experiments). *Lower:* Uptake of [^{3}H]-ouabain by the plasma-vitelline membrane complex expressed as % total [^{3}H]-ouabain taken up by the oocyte. Physiological $[K^+]_o$, o—o; high $[K^+]_o$, ●—●

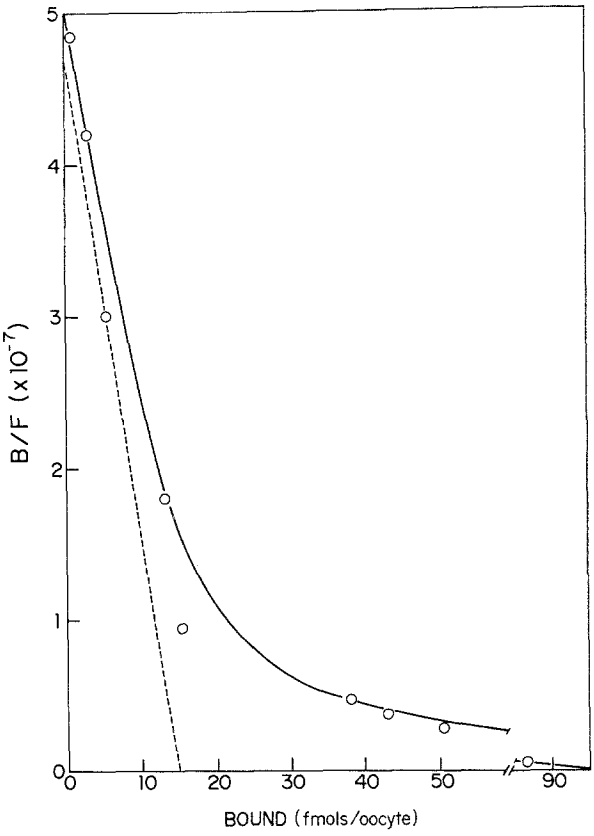

Fig. 3. Scatchard plot of [^{3}H]-ouabain binding to plasma-vitelline membrane complex of denuded prophase oocytes. Uptake was measured after 60 min at 20–22 °C in the presence of varying amounts of [^{3}H] ouabain ± 100-fold excess unlabeled ouabain. The solid line was generated assuming two classes of binding sites with K_d's and capacities, respectively, of 3.2×10^{-8} M and 15 fmol, and 2.0×10^{-6} M and 80 fmol. The dashed line shows the contribution of the high-affinity sites (3.2×10^{-8} M K_d) to the Scatchard curve

when strophanthidin is added. A series of voltage-clamp experiments done in 9.5 mM K^+ Ringer's showed a strophanthidin-sensitive membrane current of 180 ± 17 nA (mean ± SE, $n=5$) as compared to 91 ± 12 nA ($n=4$) for control oocytes in the standard Ringer's solution. Third, as shown in Fig. 1, 10^{-5} M strophanthidin inhibited both the uptake of $^{42}K^+$ from the medium and the efflux of $^{22}Na^+$ from prophase-arrested oocytes. Calculations based on these data indicate a coupling ratio of between 3 Na^+:2 K^+ and 2 Na^+:1 K^+ for the Na^+, K^+-pump (*see* Fig. 1, legend) and an electrogenic current of between 65 and 100 nA per oocyte, a range consistent with that measured electrophysiologically. Fourth, strophanthidin inhibited Na^+, K^+-ATPase activity in homogenates of prophase oocytes by about 70% (Table 3). By the end of the first meiotic division, endogenous Na^+, K^+-ATPase activity had decreased five- to six-fold and the addition of 1.0 mM strophanthidin actually stimulated Na^+, K^+-ATPase activity sevenfold.

Changes in [^{3}H]-Ouabain Uptake by the Vitelline-Plasma Membrane Complex during the First Meiotic Division

Denuded prophase-arrested oocytes were incubated in Ringer's solution containing 8.6×10^{-8} M [^{3}H]-oua-

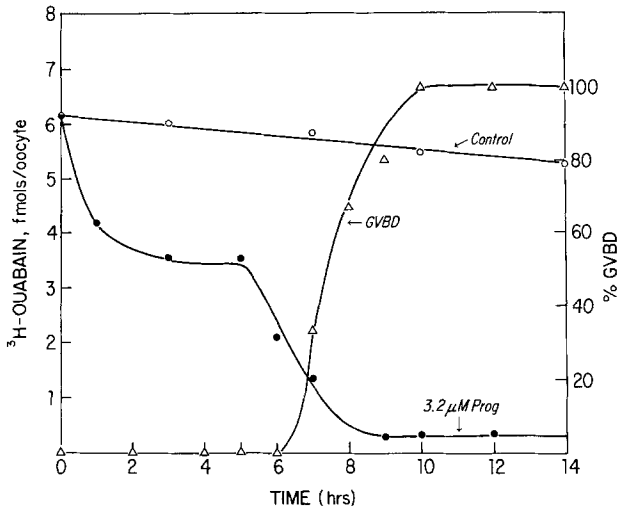

Fig. 4. Comparison of [^{3}H] ouabain binding to the plasma-vitelline membrane complex and time course of nuclear (GVBD) breakdown in denuded oocytes. [^{3}H]-Ouabain uptake during 30-min pulses in unstimulated oocytes (○—○) and oocytes during continuous exposure to 3.2 M progesterone (●—●). (△—△) Time course of germinal vesicle (nuclear) breakdown

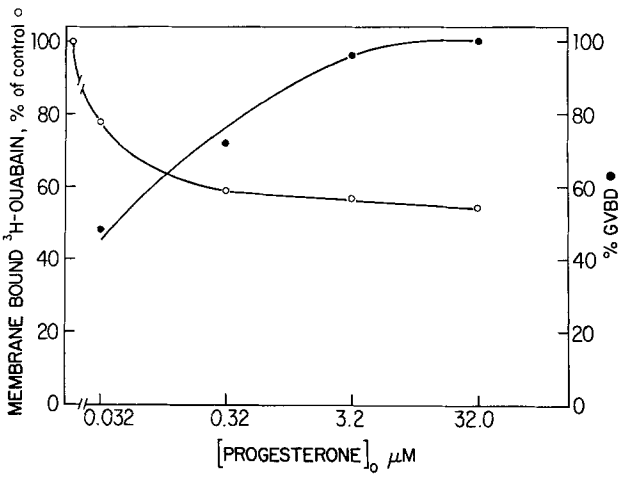

Fig. 5. Effect of progesterone preincubation on [^{3}H]-ouabain uptake by the plasma-vitelline membrane complex. *Left-hand ordinate:* Membrane bound [^{3}H]-ouabain in progesterone-treated oocytes as % of [^{3}H]-ouabain bound by untreated sibling oocytes (○—○). *Right-hand ordinate:* % nuclear breakdown (GVBD, ●—●). Denuded oocytes were preincubated with the progesterone concentration indicated for 1 hr prior to exposure to Ringer's solution containing 9×10^{-8} M [^{3}H]-ouabain plus the same [progesterone]$_o$ for 30 min

bain, and the vitelline-plasma membrane complex was isolated at 5, 15, 30, and 60 min. The ouabain concentration used was about one hundredth of that required for strophanthidin-inhibition of the *in situ* Na^+, K^+-ATPase. As shown in the upper part of Fig. 2, membrane uptake approached a plateau by 60 min. Maximal uptake was about 10 fmol/oocyte or about 3.1 nmol kg^{-1} wet wt (3.2 ± 0.1, mg wet wt per oocyte, mean ± SD, $n=4$). As shown in the lower part of Fig. 2, membrane-associated [^{3}H]-ouabain accounted for nearly 80% of the total uptake during the first 5–15 min and plateaued at about 65% after 30 min. About 0.5% of the total [^{3}H] was recovered in the nucleus by 30 min with the remaining 35% in the cytoplasm. [^{3}H]-ouabain accumulation by the oocyte membrane was inhibited by elevated (113 mM) K^+ concentrations in the medium. Increasing $[K^+]_o$ from 1.9 to 9.5 mM inhibited [^{3}H]-ouabain binding by 75% with maximal inhibition (85%) at 40–50 mM $[K^+]_o$. K_i was found to be about 5 mM $[K^+]_o$.

Figure 3 illustrates a Scatchard type plot of [^{3}H]-ouabain binding to prophase oocyte membranes. Denuded oocytes were incubated with various ouabain concentrations for 60 min and membranes were isolated as described in Methods. Bound ouabain is expressed as fmol per oocyte membrane. A plot of B/F *vs.* B indicates a single class of binding sites with a $K_d \simeq 3 \times 10^{-8}$ M at ouabain concentrations below 10^{-6} M and additional low affinity site(s) at concentrations above 10^{-6} M. Mean values ± SEM for five experiments were $4.2 \pm 0.9 \times 10^{-8}$ M and 16 ± 2 for K_d and fmol bound per oocyte, respectively. Assuming a 1:1 relationship between ouabain binding and membrane binding sites, the x intercept indicates about 1×10^{10} "high affinity" sites per oocyte membrane. For an oocyte 1.8 mm in diameter there would be a maximum of about 1,000 ouabain binding sites per μm^2.

In contrast to the prophase oocyte, oocytes completing the first meiotic division (18–24 hr) accumulated only 0.19 ± 0.05 (mean ± SEM, $n=4$) fmol [^{3}H]-ouabain per oocyte membrane in 60 min. This uptake represents only about 2% of that taken up by the prophase-arrested oocyte (Fig. 2). To determine if [^{3}H]-ouabain uptake by the oocyte membrane changed at specific time point(s) during meiosis, oocytes were continuously exposed to induced levels (3.2 μM) of progesterone and [^{3}H]-ouabain uptake was measured using 30-min pulses at intervals during completion of the first meiotic division. As illustrated in Fig. 4, there was an apparent decrease in [^{3}H]-ouabain uptake by the oocyte membrane during the first hr followed by a subsequent decrease during nuclear (*GVBD*) breakdown. [^{3}H]-Ouabain uptake by untreated (control) oocytes remained relatively constant over the 14 hr shown.

Since [^{3}H]-ouabain uptake by oocytes undergoing meiosis was measured in the presence of 3.2 μM progesterone, competition studies were carried out between steroid (progesterone) and sterol (ouabain). Denuded oocytes were preincubated for 1 hr in 0.032, 0.32, 3.2, and 32.0 μM progesterone concentrations and then exposed to 8.6×10^{-8} M [^{3}H]-ouabain plus the same [progesterone]$_o$ for 30 min. As shown in Fig. 5, 0.032 μM progesterone reduced [^{3}H]-ouabain

Table 4. [^{3}H]-Leucine uptake and incorporation into the *Rana* oocyte during the first meiotic division

Time (hr)	Nuclear breakdown (%)	[^{3}H]-Leucine (dpm/oocyte)					
		Membrane complex		Cytoplasm, TCA soluble		Cytoplasm, TCA Ppt.	
		Control	Prog.	Control	Prog.	Control	Prog
1	0	39	57	7,760	7,050	396	350
2	0	102	70	11,600	8,570	1,130	760
5	0	346	350	14,900	14,000	2,480	1,820
8	0	472	510	14,200	13,600	4,220	3,390
10	90	541	7,470	12,300	12,600	8,190	5,370
16	100	894	5,810	14,900	12,800	6,240	4,610

At different times after denuded oocytes were placed in Ringer's containing [^{3}H]-L-leucine without control) or with 1.65 μM progesterone (prog.), oocytes were removed and processed as described in the text.

uptake by the membrane with maximal inhibition of [^{3}H]-ouabain uptake at about 0.32 μM. Interestingly, 50% competition with [^{3}H]-ouabain binding coincided with 50% response at [progesterone]$_0$ of 0.032 μM. Thus, the initial decrease in [^{3}H]-ouabain uptake by the oocyte membrane seen in Fig. 4 appears to be due to competition for progesterone binding sites.

Changes in [^{3}H]-L-Leucine Incorporation during the First Meiotic Division

The virtually complete disappearance of both the strophanthidin-sensitive membrane current and [^{3}H]-ouabain binding at about the time of nuclear breakdown prompted us to examine the metabolic integrity of the oolemma. Table 4 shows the results of an experiment in which the time course of [^{3}H]-leucine uptake and incorporation were compared in untreated (control) and progesterone-induced (1.6 μM) oocytes prior to, during, and after nuclear breakdown. In this experiment, nuclear breakdown and loss of [^{3}H]-ouabain binding occurred at 8–10 hr. Oocytes were collected at the times indicated, the vitelline-plasma membrane complex was removed, rinsed as described in Methods, and [^{3}H] measured. Cytoplasmic proteins were precipitated with 6% TCA and compared with the TCA-soluble [^{3}H]. When expressed as % of the control oocytes, [^{3}H]-leucine incorporation into oocyte membranes increased more than 13-fold at about the time of nuclear breakdown (8–10 hr) with no significant change in [^{3}H]-leucine uptake into the acid-soluble pool. In three such experiments, progesterone induced [^{3}H]-leucine uptake and/or incorporation by 940% ± 280, 81% ± 23, and 98% ± 8 for membranes, cytoplasmic proteins, and TCA-soluble, respectively, by the completion of nuclear breakdown. 17β-Estradiol (a noninducer) did not affect [^{3}H]-leucine uptake and/or incorporation. Only about 5–6% of the membrane-associated [^{3}H]-leucine could be extracted with 6% TCA; 60–70% of the extracted [^{3}H] could be recovered by co-precipitation with unlabeled oocyte protein. In the control (prophase) oocytes, 5.4% ± 2.2 (mean ± SEM, $n=4$) of the total acid-precipitable [^{3}H]-leucine-labeled protein was recovered with the membrane fraction, whereas 55.1% ± 2.1 of the total [^{3}H]-labeled protein was associated with the oocyte membrane following nuclear breakdown.

Discussion

The results reported here indicate that the plasma membrane potential of the amphibian oocyte in prophase arrest is in large part due to an electrogenic Na^+, K^+-pump and that this electrogenic pump is down-regulated in response to progesterone during the first meiotic division. The disappearance of the strophanthidin-sensitive membrane current, K^+ conductance, Na^+, K^+-ATPase activity, and [^{3}H]-ouabain binding to the plasma membrane complex appears to coincide with plasma membrane depolarization [27] as well as with the swelling and breakdown of the oocyte nucleus that occurs 8–10 hr after the hormonal stimulus for resumption of meiosis.

An examination of [^{3}H]-ouabain binding to the plasma-vitelline membrane complex of the prophase oocyte indicates at least two components: a K^+-sensitive, high affinity site ($K_d = 4.2 \times 10^{-8}$ M) linked to inhibition of the pump, and a low affinity site which may not necessarily be associated with transport inhibition. This differential sensitivity is suggested by the finding that [^{3}H]-ouabain uptake by the membrane continued to increase when (i) the glycoside concentration was increased beyond that causing maximal transport ($^{42}K^+$, membrane current) inhibition, and (ii) exposure times longer than those required (1–2 min) to produce full inhibition were used.

The apparent K_d (4.2×10^{-8} M) and site density (1000/μm^2) of the [^{3}H]-ouabain binding described here are somewhat lower than that reported for muscle [4]. High affinity [^{3}H]-ouabain binding disappeared at about the time of nuclear breakdown (Fig. 4). Furthermore, [^{3}H]-ouabain taken up by the membrane complex prior to nuclear breakdown appeared both in the medium and oocyte cytoplasm at about the time of nuclear breakdown, suggesting that the K^+ site at the outer surface of the oocyte membrane, in effect, disappears. This could be due to changes in protein conformation and/or translocation of the catalytic subunit of the pump ATPase into the cytoplasm.

Progesterone appears to act at the oocyte surface to reinitiate the meiotic divisions [5, 6, 22]. An interesting finding was that progesterone inhibited [^{3}H]-ouabain binding to the plasma-vitelline membrane complex (Fig. 5). Maximal inhibition of ouabain binding occurred at progesterone concentrations essentially equal to that of ouabain. However, only 40–45% of the [^{3}H]-ouabain binding was blocked by preincubation with increasing $[progesterone]_o$ and suggests that both progesterone-sensitive and progesterone-insensitive ouabain binding sites are present on the membrane Na^+, K^+-ATPase. Both possible "sites" appear to be K^+-sensitive and have a similar K_d since only a single K_d was apparent and 40–50 mM K^+ displaced 80–85% of the bound [^{3}H]-ouabain. Although this would suggest a possible direct action of progesterone on the Na^+, K^+-pump, no significant change in membrane current was seen from 0 to 60 min following addition of progesterone to the prophase oocyte. Progesterone does have an early action that includes the release of Ca^{2+} from the oocyte surface during the first minute [20] and a down-regulation of membrane adenylate cyclase within the first 15–30 min [9]. In any case, it is apparent that progesterone induces the depolarization of the oocyte plasma membrane which begins at about the time of nuclear breakdown. Interestingly, both of these events can be prevented by treating oocytes with the protein synthesis inhibitor, cycloheximide [27]. Thus, it appears that properties of the nuclear and plasma membrane are under translational control.

One known translational event is the appearance of a cytoplasmic protein factor within the first few hours after exposure to hormones releasing the prophase block [12, 25]. This protein factor (called "maturation promoting factor" or "MPF") when injected in small quantities into prophase-arrested oocytes initiates nuclear breakdown and arrest at second meiotic metaphase [12]. The appearance of this factor can be blocked by cycloheximide [25], and it is produced even in the absence of the oocyte nucleus [12]. Previous studies in this laboratory with *Rana* oocytes [26] and by Lee and Steinhardt [10] with *Xenopus* follicles have shown that transfer of cytoplasm from oocytes undergoing the first meiotic division into prophase-arrested oocytes results in both nuclear breakdown and plasma membrane depolarization identical to that measured after hormone-stimulation. Transfer of cytoplasm from prophase-arrested *Rana* oocytes was without effect [26]. The two events were again found to be coupled temporally, and suggest that the same cytoplasmic factor(s) may be altering both plasma and nuclear membrane properties. A role for MPF in plasma membrane depolarization would explain the protein synthesis requirement, and suggests that this protein factor may down-regulate the Na^+, K^+-pump ATPase as well as turn off K^+ conductance of the plasma membrane. Studies are in progress to determine the effect of purified MPF on Na^+, K^+-ATPase activity in isolated oocyte plasma-vitelline membranes.

A second known translational event in *Rana* is a marked increase in protein synthesis at about the time of nuclear breakdown [23, 17]. As shown here, this newly synthesized protein appears to be largely associated with the plasma-vitelline membrane complex; [^{3}H]-leucine incorporation increased about ninefold in the membrane complex with no significant change in the acid-soluble pool or in incorporation into cytoplasmic proteins. The relationship between MPF production and synthesis of this membrane protein is not clear. We have also shown [7] saturable and specific binding ($K_d = 2.8 \times 10^{-8}$ M) of the nonmetabolizable progestin R5020 to macromolecules in the prophase oocyte cytosol. As discussed elsewhere [7], Molinari et al. [13] have proposed that the "native" cytosol receptor may exert a regulatory function in the initiation of protein synthesis in the extranuclear compartment. This mechanism would be consistent with the finding that progesterone induces both MPF production and membrane depolarization and that both events are cycloheximide sensitive and occur in enucleated oocytes. Further studies are necessary to determine if the increase in membrane protein synthesis (Table 4) occurs in the absence of the nucleus.

In summary, progesterone appears to act at the oocyte surface to initiate events that lead to the synthesis of a cytoplasmic protein factor or factors which in turn results in the down-regulation of the electrogenic Na^+, K^+-pump and depolarization of the oocyte plasma membrane. Depolarization coincides with a marked increase in protein synthesis and/or turnover in the plasma membrane and is indicative of major changes in membrane structure and function. Membrane depolarization and structural changes may be essential for the development of an excitable state

as a prerequisite for fertilization. It should be emphasized that the depolarization which occurs during nuclear breakdown is only one of a series of electrical and permeability changes during the meiotic divisions. A positive-going hyperpolarization occurs following sperm entry [15, 18]; the oocyte then repolarizes (inside negative), and the ouabain-sensitive Na^+, K^+-ATPase activity reappears during the first mitotic (cleavage) division [15, 16]. Thus, the techniques and model system used here should prove to be very useful for investigating the mechanism(s) of up- as well as down-regulation of this ubiquitous plasma membrane-bound enzyme.

This research was supported in part by National Institutes of Health grant HD-10463. S.P.W. was supported by Training Grant 2T32 GM 7288.

References

1. Belanger, A.M., Schuetz, A.W. 1975. Precocious induction of activation responses in amphibian oocytes by divalent ionophore A23187. *Dev. Biol.* **48**:378–381
2. Cross, M.H., Cross, P.C., Brinster, R.L. 1973. Changes in membrane potential during mouse egg development. *Dev. Biol.* **33**:412–416
3. Dumont, J.N., Brummett, A.R. 1978. Oogenesis in *Xenopus laevis* (Daudin): V. Relationships between developing oocytes and their investing follicular tissues. *J. Morphol.* **155**:73–98
4. Erlij, D., Grinstein, S. 1976. The number of sodium ion pumping sites in skeletal muscle and its modification by insulin. *J. Physiol. (London)* **259**:13–31
5. Godeau, J.F., Schorderet-Slatkine, S., Hubert, P., Baulieu, E.E. 1978. Induction of maturation in *Xenopus laevis* oocytes by a steroid linked to a polymer. *Proc. Natl. Acad. Sci. USA* **75**:2353–2357
6. Ishikawa, K., Hanaoka, Y., Kondo, Y., Imai, K. 1977. Primary action of steroid hormone at the surface of amphibian oocyte in the induction of germinal vesicle breakdown. *Mol. Cell. Endocrinol.* **9**:91–100
7. Kalimi, M., Ziegler, D., Morrill, G.A. 1979. Characterization of a progestin-binding macromolecule in the amphibian oocyte cytosol. *Biochem. Biophy. Res. Commun* **86**:560–567
8. Keynes, R.D., Swan, R.C. 1959. The permeability of frog muscle fibres to lithium ions. *J. Physiol. (London)* **147**:626–638
9. Kostellow, A.B., Ziegler, D., Morrill, G.A. 1980. Regulation of Ca^{2+} and cyclic AMP during the first meiotic division in amphibian oocytes by progesterone. *J. Cyclic Nucleotide Res.* **6**:347–358
10. Lee, S.C., Steinhardt, R.A. 1981. pH changes associated with meiotic maturation in oocytes of *Xenopus laevis*. *Dev. Biol.* **85**:358–369
11. Masui, Y. 1967. Relative roles of the pituitary, follicle cells and progesterone in the induction of oocyte maturation in *Rana pipiens*. *J. Exp. Zool.* **117**:365–376
12. Masui, Y., Markert, C.L. 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**:129–146
13. Molinari, A.M., Medici, N., Moncharmont, B., Puca, C.A. 1977. Estradiol receptor of calf uterus: Interaction with heparin-agarose and purification. *Proc. Natl. Acad. Sci. USA* **74**:4886–4890
14. Moreau, M., Guerrier, P., Dorée, M. 1976. Modifications précoces des proprietés électriques de la membrane plasmique des ovocytes de *Xenopus laevis* au cours de la réiniation meiotique induite par la progestérone, la parachloromercuribenzoate (pCMB) ou l'ionophore A23187. *C.R. Acad. Sci. Paris D.* **282**:1309–1312
15. Morrill, G.A., Kostellow, A.B., Murphy, J.B. 1971. Sequential forms of ATPase activity correlated with changes in cation binding and membrane potential from meiosis to first cleavage in *R. pipiens*. *Exp. Cell Res.* **66**:289–298
16. Morrill, G.A., Kostellow, A.B., Murphy, J.B. 1975. Role of Na^+, K^+-ATPase in early embryonic development. *Ann. N.Y. Acad. Sci.* **242**:543–559
17. Morrill, G.A., Schatz, F., Zabrenetzky, V.S. 1975. RNA and protein synthesis during progesterone-induced germinal vesicle breakdwon in *R. pipiens* ovarian tissue. *Differentiation* **4**:143–152
18. Morrill, G.A., Watson, D.E. 1966. Transmembrane electropotential changes in amphibian eggs at ovulation, activation and first cleavage. *J. Cell. Physiol.* **67**:85–92
19. Morrill, G.A., Ziegler, D. 1980. Na^+ and K^+ uptake and exchange by the amphibian oocyte during the first meiotic division. *Dev. Biol.* **74**:216–223
20. Morrill, G.A., Ziegler, D.H., Kostellow, A.B. 1980. Kinetics of calcium efflux and exchange from *Rana pipiens* oocytes immediately following reiniation of the first meiotic division: Comparison of various meiotic agonists and antagonists. *Cell Calcium* **1**:359–370
21. Petzelt, C., 1970. Ca^{2+}-activated ATPase during the cell cycle of the sea urchin *Strongylocentrotus purpuratus*. *Exp. Cell Res.* **70**:333–339
22. Smith, L.D., Ecker, R.E. 1969. Role of the oocyte nucleus in physiological maturation in *Rana pipiens*. *Dev. Biol.* **19**:281–309
23. Smith, L.D., Ecker, R.E., Subtelny, S. 1966. The initiation of protein synthesis in eggs of *Rana pipiens*. *Proc. Natl. Acad Sci. USA* **56**:1724–1728
24. Wallace, R.A., Steinhardt, R.A. 1977. Maturation of *Xenopus* oocytes: II. Observations on the membrane potential. *Dev. Biol.* **57**:305–316
25. Wasserman, W.J., Masui, Y. 1975. Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in *Xenopus* oocytes. *Exp. Cell. Res.* **91**:381–388
26. Weinstein, S.P., Ziegler, D., Kostellow, A.B., Morrill, G.A. 1981. Sequential changes in plasma membrane properties of *Rana* oocytes during the first meiotic division. *Fed. Proc.* **40**:611
27. Ziegler, D., Morrill, G.A. 1977. Regulation of the amphibian oocyte plasma membrane ion permeability by cytoplasmic factors during the first meiotic division. *Dev. Biol.* **60**:318–325

Received 3 November 1981; revised 2 March 1982