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Selective Protein Transport: Characterization and Solubilization of the Phosvitin Receptor From Chicken Oocytes

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Phosvitin (PV), a subunit of a female-specific protein, vitellogenin, binds to oocyte membranes with a KD of 10^{-6} M. Binding reaches equilibrium within 30 min after incubation at 25°C. Bound ^{125}I-PV dissociates from the membrane with a $t_{1/2}$ of 13 h when incubated in buffer. However, when ^{125}I-PV-labeled membranes are incubated in buffer containing 10^{-5} M unlabeled PV, 50% of the initially bound ^{125}I-PV dissociates from the membrane within 10 min. These results support the conclusion that PV binds to a membrane-associated receptor.

Solubilization studies show that Triton X-100 solubilizes up to 45% of the total membrane-bound ^{125}I-PV. Gel-exclusion chromatography of the solubilized material yields a 500,000 dalton ^{125}I-PV-containing complex separated from free ^{125}I-PV. The 500,000 dalton complex completely dissociates to yield free ^{125}I-PV when incubated with excess unlabeled PV. However, when incubated with 1) no addition, 2) IgG, or 3) serum albumin, the extent of dissociation is significantly reduced and is consistent with that which would be predicted on the basis of the observed dissociation rate in the absence of unlabeled PV. These results suggest that bound ^{125}I-PV can only be displaced by unlabeled PV.

These results also indicate that the 500,000 dalton species is a solubilized PV-receptor complex and that it is possible to solubilize the PV-receptor in an active form.

Key words: oocyte protein transport, receptor solubilization, phosvitin receptor

Selective protein transport mediated by coated pits and coated vesicles appears to be a ubiquitous biologic process and is particularly manifest in oocytes sequestering vitellogenin. Vitellogenin is a maternal serum protein which is stored in the oocyte until needed in embryogenesis, at which time it is degraded to provide nutrients for the developing embryo. Detailed studies suggesting that coated pits and coated vesicles mediate the transport of vitellogenin have been carried out in the Mosquito [1], in Xenopus laevis [2, 3], in Saturnid moth [4], in Cecropia moths [5], and in the domestic chicken [6]. In addition to vitellogenin, immunoglobulins are also apparently transported from the mother to her offspring by coated pits and coated vesides [7]. This maternofetal

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transport of immunoglobulins has been postulated by Brambell [8] to provide the newborn with passive immunity to its septic environment until it is immunocompetent. Specific examples include IgG transport across the chicken oocyte membrane [9], IgG transport across the chicken oocyte membrane [9], IgG transport into rabbit fetuses via the yolk sac [10], and IgG transport across rat illial cells [11]. Coated pits and coated vesicles have also been shown to mediate protein transport in systems unrelated to oogenesis or reproduction. Low-density lipoprotein, a serum cholesterol carrier, appears to enter fibroblasts exclusively via coated vesicles [12]. Epidermal growth factor also appears to enter cells via a coated-vesicle-mediated mechanism [13].

Proteins appear to be selected for specific transport by receptors which recognize and bind specific proteins from the extracellular millieu. Receptors have been characterized which mediate binding in a variety of protein transport systems. Examples include the vitellogenin receptor on chicken oocyte membranes [14], the vitellogenin receptor on Xenopus oocytes [15], IgG receptors on chick yolk sac [16], IgG receptors on rabbit yolk sac [17, 18], and IgG receptors on rat illial cells [19]. In addition, receptors have been characterized for low-density lipoprotein [20] and epidermal growth factor [21] on fibroblasts. The techniques used to study the receptors involved in protein transport were taken from prior studies of receptors for insulin [22], IgE [23], acetylcholine [24], and other molecules. For a detailed review on the development and use of these methods, see Cuatrecasas and Hollenberg [25].

The objective of this study is to elucidate further the mechanism of vitellogenin transport into developing chicken oocytes. We present evidence that phosvitin, a subunit of vitellogenin, is selectively bound to receptors on developing oocytes. We also present evidence suggesting that Triton X-100 solubilizes the phosvitin receptor in an active form.

MATERIALS

Agarose 0.5 M, Agarose 5 M, and Bio Gel P-10 were obtained from Bio-Rad Laboratories. Carrier-free $Na^{125}I$ was obtained from the Amersham Radiochemical Centre. Bovine serum albumin (BSA), chicken serum albumin, and phosvitin (PV) were from Sigma Co. Siliclad was obtained from Clay Adams. All other chemicals were of reagent grade and were obtained from commercial sources. Live White Leghorn laying hens and oocytes were purchased from a local slaughter house.

Chicken IgG, kindly provided by Dr. Carol Linden, was purified from egg yolks using a modification of the method of Bernardi and Cook [16, 26].

All experimental procedures were carried out in an incubation buffer (IB) consisting of 0.01 M Mes (2-N-morpholinoethane sulfonic acid), pH 6.0, 0.14 M NaCl, 5 mM KCl, 0.83 mM $MgSo_4$, 0.13 mM $CaCl_2$, plus 0.02% sodium azide. Where indicated, bovine serum albumin was added to a final concentration of 5 mg/ml (IB-BSA).

METHODS

Iodination of Phosvitin

Phosvitin was iodinated by the procedure of Pressman and Eisen [27]. In a typical iodination, 5 mCi of $Na^{125}I$ were first oxidized to $^{125}ICl^-$ with $NaNO_2$ in 1 M HCl. The pH of the solution was then adjusted to neutrality and 10 mg of PV was added in 0.1 M borate buffer, pH 8.0. After the reaction was allowed to proceed for 4 min at 0°, the reac-

tion was quenched with sodium meta-bisulfite, and ^{125}I-PV was separated from other reactants by chromatography on a Bio Gel P-10 column that had been prerun with unlabeled PV. These procedures routinely yielded ^{125}I-PB preparation with specific activities of approximately 10^8 cpm/mg.

Determination of Phosvitin Concentration

Phosvitin concentration was determined by absorbance at 280 nm using a value of $E^{1\%}_{1cm} = 4.4$, obtained by measuring the OD_{280} of various dilutions of 10 mg/ml solutions of PV. The 10 mg/ml solutions were prepared by weighing lyophilized PV. Molar concentrations were based on an average molecular weight of 34,000 daltons for the two classes of phosvitin [30].

Isolation of Oocyte Membranes

The ovaries were removed from freshly killed White Leghorn laying hens and placed in IB-BSA at 0°C. Oocytes approximately 1.5–2 cm in diameter were slit, drained of yolk and returned to ice-cold IB-BSA. Adherent yolk was removed by gentle shaking in the IB-BSA solution. The membrane complex consisting of the oocyte plasma membrane, a fibrous viteline layer, a monolayer of follicular epithelium cells, and an acellular basement lamella was dissected free of the overlaying connective tissue and placed in fresh IB-BSA. Dissected membranes were homogenized with 2–3 passes of a loose-fitting pestle in a glass homogenizer. Tissue fragments were harvested by centrifugation for 5,000 g-min at 4° and resuspended in a minimal volume of IB-BSA. In a typical experiment, 36 oocytes were dissected and the homogenized membranes resuspended in a final volume of 3.6 ml such that 100 μl of homogenate was approximately equivalent to the tissue obtained from one oocyte.

Assay of PV Binding to Oocyte Membranes

Siliclad-coated glass tubes (12 × 75 mm) were used for all incubations. In a typical binding experiment, 100 μl of homogenized oocyte membranes, an amount equivalent to the membranes from one oocyte, were placed in a tube. ^{125}I-PV and other proteins, when appropriate, were added to yield a final volume of 250 μl. Except where noted in the figure legends, incubations were carried out for 60 min in a shaking water bath at 25°C. Details of protein concentration are given in the figure legends. Incubations were routinely terminated by the addition of 3 ml of IB-BSA at 4°, followed by centrifugation for 5,000 g-min at 4° to harvest the membranes. The membrane pellet was resuspended in successive 3 ml volumes of IB-BSA and washed as described above a total of five times. ^{125}I radioactivity in the wash supernatants and final pellets was determined in a well-type gamma counter.

In our experiments, specific ^{125}I-PV binding was defined as that which was displaced by 50-fold to 100-fold molar excess of unlabeled PV, an amount sufficient to displace 98–99% of the specifically bound ^{125}I-PV. Each experiment was conducted using parallel sets of incubations, the first containing only ^{125}I-PV plus homogenized oocyte membranes and the second containing ^{125}I-PV, membranes, and unlabeled PV. The bound ^{125}I-PV resulting from the first set of tubes represents total ^{125}I-PV binding; the bound ^{125}I-PV in the second set of tubes represents nonspecific ^{125}I-PV binding. By subtracting the nonspecific from the total radioactivity bound, we arrive at values for ^{125}I-PV specifically bound. In these experiments nonspecific ^{125}I-PV binding typically represents 15–20% of the total ^{125}I-PV binding.

Solubilization Procedure

Except as noted in the figure legends, solubilization procedures were carried out as follows. Homogenized oocyte membranes obtained from a single oocyte were suspended in 333 μl of 1% (w/v) Triton X-100 in IB-BSA and incubated for 30 min at 25°. Following incubation, three samples were pooled to yield 1 ml, and insoluble material was removed by centrifugation at 100,000 g for 30 min at 4°.

Column Chromatography and Solubilized Receptors

Siliclad-treated glass columns were filled with Agarose 0.5 M (0.8 × 32 cm column) or Agarose 5 M (0.9 × 53 cm column) and equilibrated with IB containing 0.1% (w/v) Triton X-100 and 0.1 mg/ml bovine serum albumin at 4°. Elution with buffer of the same composition was carried out at a flow rate of 4 ml/h for the Agarose 0.5 M columns and 2 ml/h for the Agarose 5 M columns. Columns were routinely calibrated with ferritin (MW 480,000), bovine IgG (MW 160,000), and bovine serum albumin (MW 64,000).

RESULTS

Characterization of Phosvitin Binding to Oocyte Membranes

Association kinetics. An initial series of experiments was designed to determine the kinetic properties of phosvitin (PV) binding to homogenized oocyte membranes. To determine the rate at which PV binding reached equilibrium, identical aliquots of homogenized oocyte membranes were incubated with a low concentration (3.8×10^{-7} M) of ^{125}I-VP at 25° for various times (Fig. 1). Specific binding increased rapidly for the first 20 min of incubation; after 30 min there was no further increase. Nonspecific binding

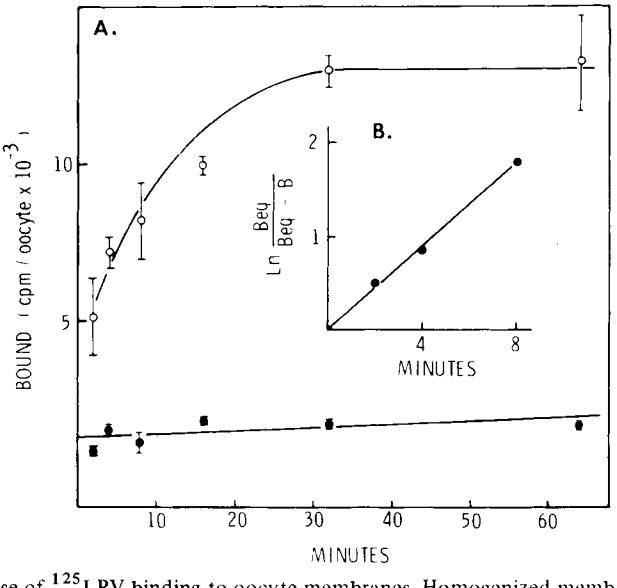

Fig. 1. Time course of ^{125}I-PV binding to oocyte membranes. Homogenized membranes were incubated with 3.8×10^{-7} M ^{125}I-PV (10^6 cpm) for the indicated times at 25°. A: Specific (○) and nonspecific (●) binding was quantitated as described in Materials and Methods. B: Replot of early time points (see text). Error bars indicate standard deviation of triplicate determinations.

increased linearly and was less than 20% of specific binding at all times. All subsequent experiments were carried out at receptor concentrations approximately equal to that in the time course experiment and with the ligand concentration equal to or greater than that used in the time course experiment. Hence, since subsequent experiments were incubated for times greater than 60 min at 25°, the binding reactions will have reached equilibrium.

The association rate constant, k_1, was determined by the method of Kitabgi et al [28]. Originally developed to analyze the interaction between neurotension and synaptic membranes, this method assumes that the free-ligand concentration equals total ligand concentration. In our experiments, this assumption is only valid for short incubation times. Thus, according to Kitabgi [28], for short incubation times the interaction of ^{125}I-PV and oocyte membranes can be described as a pseudo-first-order reaction by the equation:

$$\ln \frac{[RL_{eg}]}{[RL_{eg}] - [RL]} = ([L]k_1 + k_{-1}) \cdot t$$

$[RL_{eg}]$ is the concentration of bound PV at equilibrium, [RL] is the concentration of bound PV at time t, [L] is the toal concentration of PV, k_1 is the association rate constant and k_{-1} is the dissociation rate constant. When $\ln \frac{[RL_{eg}]}{[RL_{eg}] - [RL]}$ was plotted as a function of time (Fig. 1B), a straight line was obtained with a slope of

$$3.8 \times 10^{-7}\ M\ (k_1) + k_{-1} = 3.7 \times 10^{-3}\ sec^{-1}$$

Dissociation kinetics. The dissociation rate of ^{125}I-PV from oocyte membranes was determined at 25° and 4° by measuring the dissociation of bound ^{125}I-PV in the presence of excess unlabeled PV. Under these conditions, the association reaction of ^{125}I-PV with the receptor is blocked and the dissociation reaction can be described by the equation

$$\frac{d[RT]}{dt} = -k_{-1}\ [RL]$$

thus,

$$k_{-1} = \frac{1}{t} \ln \frac{[RL]0}{[RL]}$$

and

$$k_{-1} = \frac{\ln 2}{t_{1/2}},$$

where $t_{1/2}$ = half-time of dissociation.

The results (Fig. 2A) show that dissociation at 4° is linear when plotted as a semilogarithmic plot of percentage bound versus time, and the $t_{1/2}$ (4°) is approximately 13 h. The dissociation rate at 25° appeared to be biphasic. When corrected for the contribution of the slow component ($t_{1/2}$ = 13 h), the data for the rapid component yielded a straight line on a semilogarithmic plot with a $t_{1/2}$ of approximately 10 min. Thus, the dissociation rate constant, (k_{-1}), equals 1.1×10^{-3} sec. Using the association kinetic data and the experimentally determined value of k_{-1}, the association rate constant was calculated as $6.8 \times 10^3\ M sec^{-1}$. From the values of the rate constants, the dissociation constant, K_D, was calculated as

$$K_D = \frac{k_{-1}}{k_{+1}} = 1.6 \times 10^{-7}$$

The presence of a slowly dissociating component at 25°, which represents about 20% of the bound ^{125}I-PV, is at present unexplained. However, since the data for the dissociation experiment was not corrected for nonspecific binding, and nonspecific binding represents 15–20% of the total ^{125}I-PV bound at the ligand concentrations used in this experiment (data not shown), the slowly dissociating component may represent dissociation of nonspecifically bound ^{125}I-PV. Alternatively, the slowly dissociating component may represent the receptor-ligand complex undergoing a transition to form a more tightly coupled receptor-ligand complex, as was proposed for neurotoxin binding to the acetylcholine receptor [24] $[R + L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RL \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} RL]$. If this is true, we have calculated the K_D only for the first half of the overall reaction: $K_D = \frac{k_{-1}}{k_1}$. Further experiments will be required to determine the significance of the slowly dissociating component.

In a similar experiment, dissociation of bound ^{125}I-PV was determined in the absence of excess unlabeled PV (Fig. 2A). In this case, reassociation of ligand and receptor is possible. This experiment was done to determine the extent of dissociation that would occur during the washing procedure used to separate free and bound ligand. At 4° in the absence of unlabeled PV, the half-time of dissociation was about 13 h. Thus, less than 5% of the bound ^{125}I-PV would be expected to dissociate from the membrane during the 30-min washing procedure at 4°C. These results also suggest that a significant amount of ^{125}I-PV receptor complexes may be expected to remain after 20 h, which is approximately the maximum time required for the column chromatography experiments in the following sections.

Displacement assay. In order to assay the ability of unlabeled PV to competitively displace bound ^{125}I-PV from oocyte membranes, an additional displacement experiment was carried out. The results (Fig. 2B) show that at the lowest concentration tested (1.1×10^{-6} M), 84% of the initially bound ^{125}I-PV remained bound after a 60-min incubation at 25°. However, at the highest concentration of unlabeled PV tested (3.9×10^{-4} M), only 19% of the initially bound ^{125}I-PV remained associated. This experiment suggests that at least 80% of the initially bound ^{125}I-PV was bound in a freely reversible manner.

Equilibrium studies. The apparent dissociation constant, K_D, of PV binding to homogenized oocyte membrane was determined under steady-state conditions. In this experiment, the concentration dependence of PV binding was assayed by adding increasing amounts of unlabeled PV to a constant amount of ^{125}I-PV and incubating with oocyte membranes for 2 h at 25°. A Scatchard plot of the data (Fig. 3) gives an apparent K_D of 3.3×10^{-6} M. The minimum number of binding sites per oocyte calculated from these data is approximately 6.5×10^{13}. This number is most likely a low estimate in view of the loss of tissue which undoubtedly occurs during the isolation and assay procedures.

Control for wash procedure. In these experiments the separation of free and bound ligand was routinely accomplished by washing the oocyte tissue with five 3-ml changes of cold (4°) IB-BSA. That this procedure removes most free ^{125}I-PV from the membranes was shown when oocyte membranes were first incubated with ^{125}I-PV and then subjected to repeated washing. The results (Fig. 4) show that successive washes remove progressively smaller proportions of the remaining activity, suggesting that free ^{125}I-PV comprises a smaller proportion of the total ^{125}I-PV remaining after each wash. After five repetitions, each subsequent was removed approximately 5% of the activity associated with the preceding pellet, suggesting that no free ^{125}I-PV remains associated with the pellet. In view of the very slow dissociation rate observed at 4° and of the fact that each wash requires only 5 min to complete, the 5% loss of activity in washes 6–10 may be due to a failure to pellet 100% of the membranes.

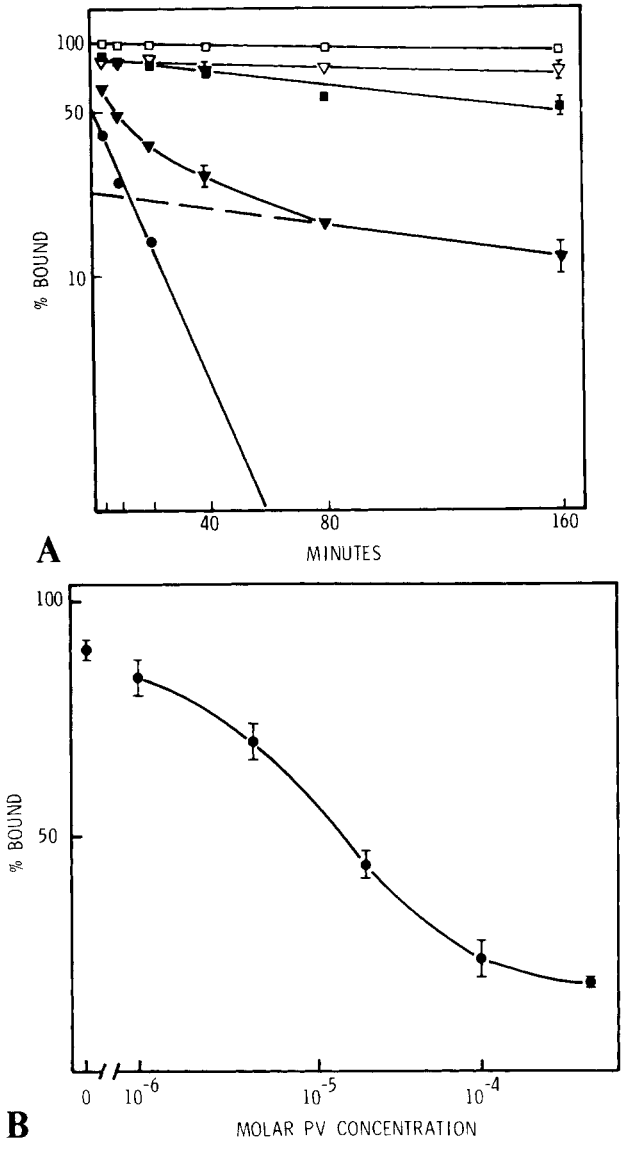

Fig. 2. Dissociation of bound ^{125}I-PV from oocyte membranes. Homogenized membranes were incubated with 3.5×10^{-6} M ^{125}I-PV (96.8×10^5 cpm) for 60 min at 25°. Unbound PV was removed by washing as described in Materials and Methods. A: Time course of dissociation of bound ^{125}I-PV. Washed membranes, containing 3×10^4 cpm of bound ^{125}I-PV were resuspended in 1 ml of IB-BSA in the presence (▼, ■) or absence (▽, □) of 3×10^{-4} M unlabeled PV and incubated for the indicated times at 25° (▼, ▽) or 4° (■, □). The fast component (●) of the 25° plus unlabeled PV data was calculated by subtraction of the contribution of the slowly dissociating component (dashed line) from the early time points. B: Displacement of bound ^{125}I-PV by unlabeled PV. Washed membranes, containing 4×10^4 cpm of bound ^{125}I-PV were resuspended in 1 ml of IB-BSA containing 0 to 3×10^4 M unlabeled PV. After incubation for 60 min at 25°, the reaction was terminated by centrifugation at 4° for 5,000 g-min. Percentage bound was calculated as: $\frac{(^{125}\text{I in pellet})}{(\text{total } ^{125}\text{I})} \times 100$. Error bars indicate standard deviations of triplicate determinations.

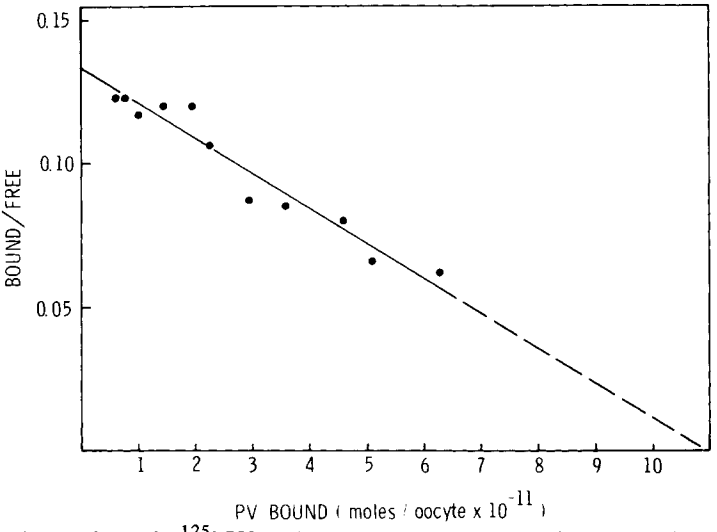

Fig. 3. Scatchard plot of specific ^{125}I-PV binding to oocyte membranes. Homogenized oocyte membranes were incubated for 2 h at 25° with a constant amount of ^{125}I-PV (3.8×10^{-7} M, 3.5×10^{5} cpm) to which increasing concentrations of unlabeled PV had been added to yield final PV concentrations of 3.8×10^{-7} M to 4.4×10^{-6} M. Bound and free ^{125}I-PV were separated by washing as described in Materials and Methods. Bound PV was that remaining with the pellet after the final wash, and free PV was the total PV recovered in the wash supernatants. Nonspecific binding, quantitated in the presence of 50 × molar excess unlabeled PV was subtracted from total bound PV. Each datum point is the mean of triplicate determinations. The best fit of the data to a straight line was calculated using linear regression analyses ($r = 0.96$) $K_D = 3.3 \times 10^{-6}$ M, number of receptors/oocyte = 6.5×10^{13}.

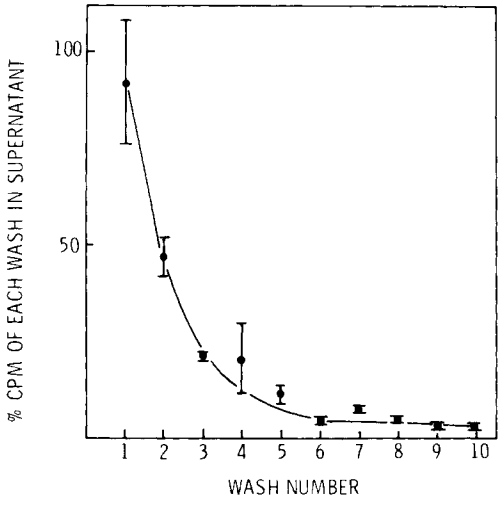

Fig. 4. Elution of free ^{125}I-PV from oocyte membranes. Homogenized oocyte membranes were incubated with 2.9×10^{-6} M ^{125}I-PV (4×10^{6} cpm) for 60 min at 25°. Following incubation, the membranes were diluted by addition of 3 ml of IB-BSA at 0°. The membranes were then harvested by centrifugation at 5,000 g-min, and the pellet was immediately resuspended in 3 ml of fresh IB-BSA for each of 10 washes. Following the final wash, 1.4×10^{5} cpm of ^{125}I-PV remained bound to the membrane pellet. Percentage cpm in each wash supernatant was calculated as the percentage of the total cpm initially present in each wash before centrifugation. Error bars indicate standard deviations of triplicate determinations.

Solubilization of Membrane-Bound ^{125}I-PV With Triton X-100

Low-speed centrifugation assay. In order to determine the degree to which Triton X-100 solubilized bound ^{125}I-PV, washed membranes containing bound ^{125}I-PV were incubated with various concentrations of Triton X-100. These experiments showed (Table 1) that Triton concentrations of 0.05–2% solubilized approximately 40–50% of the initially bound ^{125}I-PV. Triton concentrations higher than 2% appeared to solubilize only 25–35% of the initially abound material, even though more of the oocyte tissue appeared to be solubilized, since the final centrifugation yielded a much smaller pellet. The significance of this observation is not known; however, it is possible that high concentrations of Triton form large protein-mycelle aggregates which are segmentable under the conditions used in these experiments. In control experiments where membranes were incubated in the absence of Triton, only 8% of the initially bound ^{125}I-PV was released.

Gel exclusion chromatography assay. If Triton X-100 solubilizes bound ^{125}I-PV and the receptor as a complex, this PV-receptor complex should elute in a position different from free PV on an appropriate gel exclusion column. The elution profile on an Agarose 0.5 M column of material solubilized by 1% Triton X-100 from membranes incubated with ^{125}I-PV is shown in Figure 5A. The ^{125}I-PV activity eluted from the column as two peaks, one containing approximately 40% of the total activity eluted in the void volume and the other eluted in the position of free ^{125}I-PV. Because the ^{125}I-PV-containing material eluting the void volume has a higher molecular weight than free ^{125}I-PV, we shall tentatively refer to this high-molecular-weight material as solubilized PV-receptor complex.

In order to demonstrate the reversible nature of phosvitin binding to the solubilized PV-receptor complexes, these complexes were incubated in the presence or absence of excess unlabeled PV (2.9×10^{-5} M) prior to chromatography on Agarose 5 M columns.

TABLE I. Solubilization of Bound ^{125}I-PV by Triton X-100

Triton X-100 (%w/v)	% Solubilized
0.0	9.4 ± 0.7
0.01	23.4 ± 1.6
0.05	40.5 ± 0.9
0.1	45.0 ± 0.1
0.5	39.3 ± 2.5
1.0	45.0 ± 4.5
2.0	47.2 ± 0.8
3.5	33.4 ± 0.3
5.0	27.5 ± 6.4

Homogenized oocyte membranes were incubated with 5.8×10^{-6} M ^{125}I-PV ^{125}I-PV (2.4×10^{6} cpm) for 60 min at 25°. Unbound PV was removed as described in Materials and Methods. These washed membranes, containing 4×10^{4} cpm, were resuspended in 1 ml of IB-BSA with 0–5% w/v Triton X-100 and incubated for 30 min at 24°. Following incubation, the membranes were harvested by centrifugation at 4° for 5,000g-min. The percentage solubilized was calculated as: $\frac{(^{125}\text{I in supernatant})}{(\text{total } ^{125}\text{I})} \times 100$. The means of three determinations ± standard deviation are given.

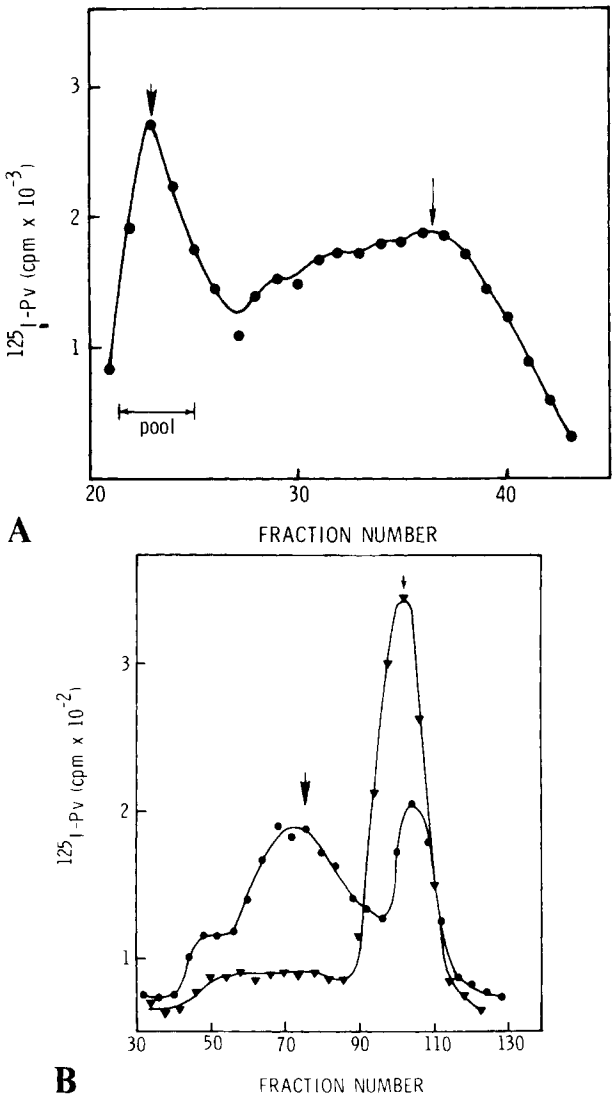

Fig. 5. Elution profiles of solubilized ^{125}I-PV receptor complexes. A: Three tubes containing 2.9 × 10^{-6} M ^{125}I-PV (94 × 10^6 cpm) were incubated for 90 min at 25° with homogenized oocyte membranes. Unbound ^{125}I-PV was removed by washing as described in Materials and Methods. The washed pellets, each of which contained 4 × 10^4 cpm, were pooled, suspended in 1 ml of 1% w/v Triton X-100 in IB-BSA, and incubated a further 30 min at 25°. Insoluble material was removed by centrifugation at 100,000 g for 30 min at 4°, and the soluble supernatant (5 × 10^4 cpm) was applied to an Agarose 0.5 M column. The larger arrow indicates void volume and the smaller arrow indicates the elution position of unbound ^{125}I-PV. B: Elution of solubilized ^{125}I-PV receptor complexes on an Agarose 5 M column. Triton-solubilized ^{125}I-PV receptor complexes (1 × 10^4 cpm) from fraction 22–25 in panel (A) above were divided into two identical aliquots and incubated with (▾) or without (•) 2.9 × 10^{-5} M ^{125}I-PV for 30 min at 25° immediately prior to application to the Agarose 5 M column. The large arrow indicates the elution position of ferritin and the smaller arrow indicates the elution position of unbound ^{125}I-PV. Incubations of ^{125}I-PV receptor complexes with either 10^{-5} M IgG or 10^{-5} serum albumen for 30 min at 25° prior to chromatography on the Agarose 5 M column resulted in elution profiles that were similar to those obtained when the ^{125}I-PV receptor complexes were incubated in the absence of unlabeled PV.

The elution profiles of ^{125}I-PV radioactivity (Fig. 5B) show that the sample incubated in the absence of unalbeled PV eluted as two peaks, both of which are well within the included volume of these columns. The major peak eluted at a position slightly larger than ferritin (480,000 daltons). The minor peak eluted at the same position as free ^{125}I-PV. In contrast, the sample which was incubated with excess unlabeled PV prior to chromatography yielded a single peak in the position of free ^{125}I-PV, indicating that unlabeled PV displaced bound ^{125}I-PV from the solubilized receptor.

The preceding experiment demonstrated that the material eluting in the void volume of an Agarose 0.5 M column contains a PV-binding component. This component must be heterogeneous, since it eluted as a broad symmetrical peak on an Agarose 5 M column. The average apparent molecular weight of this component, based on its elution position compared to ferritin and IgG, is approximately 500,000 daltons. However, since it is not known how much Triton is associated with the complex or the conformation of the complex, the true molecular weight is not known.

Additional experiments which demonstrate the specificity of the PV–receptor interaction were also carried out. Solubilized ^{125}I-PV receptor complexes were incubated with chicken serum albumin (1×10^{-5} M) or chicken IgG (1×10^{-5} M) for 30 min at 25°C and then chromatographed on Agarose 5 M columns. The results show no displacement of bound ^{125}I-PV and were similar to those obtained when ^{125}I-PV receptor complexes incubated in buffer alone were chromatographed on Agarose 5 M columns (Fig. 5B).

Demonstration of Soluble PV Receptor-Binding Activity

To determine whether Triton X-100 can solublize the PV receptor in a form capable of binding free ligand, homogenized membranes were first extracted with 1% Triton X-100 The solubilized material was assayed for PV-binding activity by incubation with ^{125}I-PV in the presence or absence of excess unlabeled PV for 60 min at 25°C, followed by chromatography on two identical Agarose 0.5 M columns (Fig. 6A). Samples incubated in the absence of excess unlabeled PV yielded a high-molecular-weight component which eluted in the void volume; however, more than 90% of eluted ^{125}I-PV was located in the same position as free ^{125}I-PV. The sample that was incubated in the presence of excess unlabeled PV eluted as a single peak in the same position as free ^{125}I-PV, indicating that no ^{125}I-PV receptor complexes had been formed.

^{125}I-PV binding to the solubilized receptor was shown to be reversible by incubation of the ^{125}I-PV receptor complexes in the presence or absence of unlabeled PV (2.9×10^{-5} M) for 30 min at 25°. Chromatography on two identical Agarose 5 M columns (Fig. 6B) indicates that the ^{125}I-PV receptor complexes incubated in the absence of unlabeled PV eluted as two peaks, one having a molecular weight slightly greater than ferritin and one eluting in the same position as free ^{125}I-PV. In contrast, the ^{125}I-PV receptor complexes, which were incubated in the presence of excess unlabeled PV prior to chromatography, eluted primarily as a single peak in the same position as free ^{125}I-PV.

The specificity of ^{125}I-PV binding to soluble receptors was demonstrated in additional experiments in which ^{125}I-PV soluble receptor complexes were incubated with excess chicken IgG (10^{-5} M) or excess chicken serum albumin (10^{-5} M) for 30 min at 25° prior to chromatography on Agarose 5 M columns. The results, which show no displacement of bound ^{125}I-PV by either IgG or albumin, are similar to that shown in Figure 6B, in which the soluble ^{125}I-PV receptor complexes were incubated with buffer alone prior to chromatography. These results suggest that only PV and not IgG or serum albumin can displace ^{125}I-PV from the solubilized receptor.

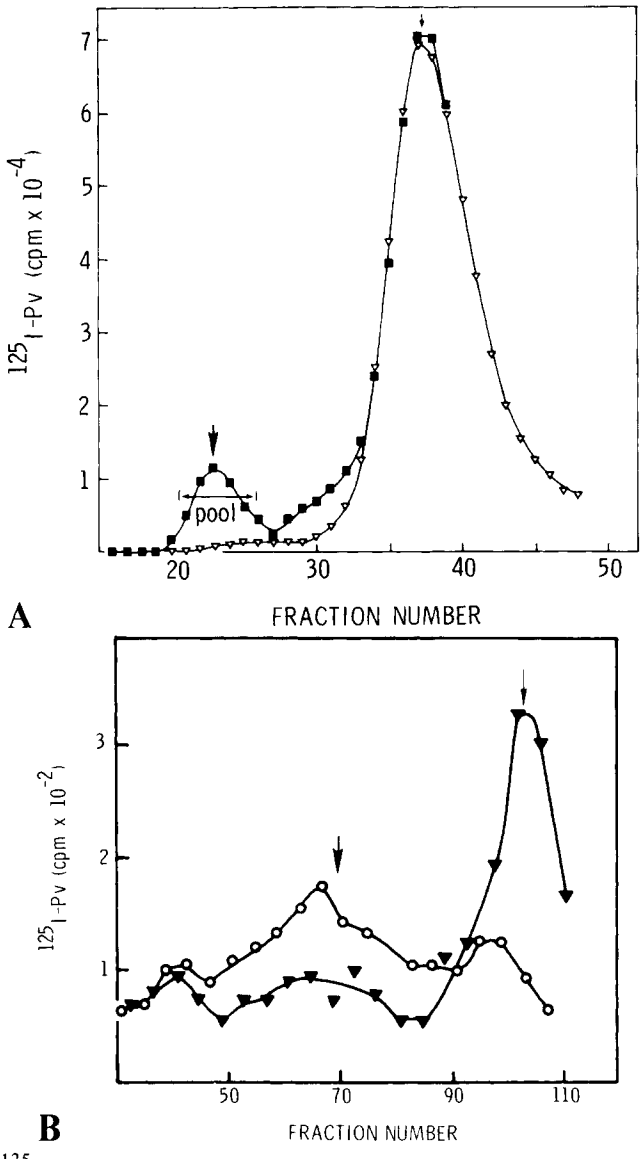

Fig. 6. Assay of ^{125}I-PV binding to previously solubilized receptors. A: Homogenized oocyte membranes from three oocytes were solubilized by incubation in 1 ml of 1% w/v Triton X-100 in IB-BSA for 30 min at 25°. Insoluble material was removed by centrifugation at 100,000 g for 30 min at 4°. The soluble supernatant fraction was divided into two equal aliquots and incubated with 5.9×10^{-7} M ^{125}I-PV (6.8×10^5 cpm) in the presence (▽) or absence (■) of 2.9×10^{-5} M unlabeled PV for 60 min at 25°. Following incubation, the samples were immediately applied to two identical Agarose 0.5 M columns. The large arrow indicates the void volume and the small arrow indicates the elution position of unbound ^{125}I-PV. B: Assay for specific displacement of ^{125}I-PV bound to soluble receptors by unlabeled PV. Soluble receptor ^{125}I-PV complexes were obtained by pooling fractions 21–25 from panel (A). This pooled material (4×10^3 cpm) was divided into two equal aliquots and incubated in the presence (▼) or absence (○) of 2.9×10^{-5} M unlabeled PV for 30 min at 25° immediately prior to chromatography on the Agarose 5 M columns. The large arrow indicates the elution position of ferritin and the small arrow indicates the elution position of unbound ^{125}I-PV. In similar experiments, incubation of soluble receptor ^{125}I-PV complexes with either 10^{-5} M, IgG, or 10^{-5} M serum albumen for 30 min at 25° prior to chromatography on Agarose 5 M columns gave elution profiles which were similar to those obtained when soluble receptor ^{125}I-PV complexes were incubated in the absence of unlabeled PV (○).

DISCUSSION

A previous report from our laboratory showed that vitellogenin binds specifically to oocyte tissue [14]. On the basis of the present data, we postulate that there are specific receptors for phosvitin on chicken oocyte membranes. PV is a subunit of a maternal serum protein, vitellogenin. In addition, we have evidence that both vitellogenin and PV bind to oocyte membranes with a K_D of about 5×10^{-7} M, and that both molecules apparently bind to the same receptor [29]. Taken together, these results suggest that the PV component of vitellogenin is recognized by the receptor and hence mediates the transport of vitellogenin into the developing oocyte.

In the present report the dissociation constant, K_D, of PV binding to oocyte membranes was determined by two separate and independent methods. One method, based on the equilibrium binding of PV and membrane-associated receptors yielded a $K_D = 3.3 \times 10^{-6}$ M. The second method, based on the independent determinations of the association rate constant k_1 and the dissociation rate constant k_{-1} yield a $K_D = 1.6 \times 10^{-7}$ M, where $K_D = \frac{k_{-1}}{k_{+1}}$. These two values are probably not significantly different, given the errors intrinsic in the assumptions made in these calculations. The K_D determined from the equilibrium experiment is probably more accurate, because fewer assumptions were made in calculating the K_D from equilibrium data.

A major finding of the present study is that phosvitin receptor-binding sites in oocyte tissue can be solubilized and assayed in the soluble state. Our assay for soluble PV-binding activity depends on the apparent increase in molecular weight as determined by gel exclusion chromatography of ^{125}I-PV when it is bound to the solubilized receptor. Free ^{125}I-PV eluted as a 80,000 dalton globular protein, whereas bound ^{125}I-PV elutes in a broad peak centered at 500,000 daltons. However, the true molecular weight of the ^{125}I-PV receptor complex is not known since the amount of Triton bound to the complex and the conformation of the complex are not known.

Gel exclusion chromatography has been used in a similar manner to assay a number of detergent-solubilized membrane-associated receptors. Specific examples include the insulin receptor [22], the IgE receptor [23], the β-adrenergic receptor [31], the parathyroid hormone receptor [32], the vasopression receptor [33], and the follitropic receptor [34].

Soluble PV-binding activity was shown to be specific. When ^{125}I-PV was incubated with solubilized oocyte membranes in the presence or absence of excess unlabeled PV, ^{125}I-PV receptor complex could be detected only in the absence of excess unlabeled PV. Specificity of binding was also shown by displacement experiments. We observed that only unlabeled PV could displace bound ^{125}I-PV (see Figs. 5B and 6B). Incubation of soluble ^{125}I-PV receptor complexes with buffer alone, serum albumin, or IgG resulted in dissociation similar to that predicted on the basis of the experiment shown in Figure 2A. The results shown in Figure 2A suggest that under similar conditions (30 min incubation, 25°, no unlabeled PV) up to 30% of the bound ^{125}I-PV would dissociate from the membrane-bound receptor. IgG and serum albumin were used in our displacement experiments because both are present in the serum and hence in vivo they would be exposed to the receptor at the same time as PV in the form of vitellogenin. In addition, IgG is specifically transported into the developing oocyte at a parallel rate to PV but at an additional developmental stage [9]. Serum albumin is apparently not transported into the oocyte. Therefore, the PV receptor must be able to discriminate between PV, IgG, and serum albumin in vivo. Our results demonstrate that the PV receptor can discriminate between IgG, albumin, and PV in vitro.

In conclusion, we have shown that a specific receptor for PV is associated with oocyte membranes. This receptor appears to mediate the specific transport of vitellogenin into the developing oocyte [28]. In addition, we have shown the PV receptor can be solubilized with the non-ionic detergent Triton X-100, and the solublized receptor retains the ability to specifically recognize PV.

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