A High Affinity Ca\(^{2+}\)-stimulated and Mg\(^{2+}\)-dependent ATPase in Rat Corpus Luteum Plasma Membrane Fractions*

(Received for publication, September 26, 1980, and in revised form, October 27, 1980)

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Plasma membrane fractions from rat corpus luteum contain two kinds of Ca\(^{2+}\)-stimulated ATPase, one having a high affinity for Ca\(^{2+}\), the other a low affinity for Ca\(^{2+}\). The high affinity ATPase had a specific Ca\(^{2+}\) requirement with a K\(_i\) of 0.2 to 0.3 mM; it had a V\(_{max}\) of 105 nmol min\(^{-1}\) mg\(^{-1}\) and distributed, upon subcellular fractionation, with recognized plasma membrane enzymes. The properties of this enzyme indicate that it is a Ca\(^{2+}\) extrusion pump. The low affinity pump (K\(_i\) for Ca\(^{2+}\), about 15 mM) was nonspecific, being stimulated equally well by Ca\(^{2+}\) or Mg\(^{2+}\); its function is unknown.

Although the high affinity ATPase resembled the erythrocyte Ca\(^{2+}\)-pumping ATPase in the properties mentioned above, it differed in that it failed to respond to Mg\(^{2+}\) or calmodulin. The lack of response to Mg\(^{2+}\) was due to the enzyme's retention of endogenous Mg\(^{2+}\); it did, after incubation with chelators, show a Mg\(^{2+}\) requirement. However, we were unable to show any effect of added calmodulin or trifluoperazine. This failure may be related to the high content of tightly bound calmodulin in these membranes. Much of this calmodulin could not be extracted even by washing with 1 mM EGTA and/or 0.1% (w/v) Triton X-100.

This enzyme, the erythrocyte enzyme, and the adipocyte plasma membrane Ca\(^{2+}\)-ATPase all belong to the class of Ca\(^{2+}\)-ATPases with plasma membrane distribution and high affinity for Ca\(^{2+}\), indicating that they are Ca\(^{2+}\) extrusion pumps. However, the data indicate that tissue-specific differences exist within this class, with the enzyme from adipocytes and rat corpus luteum belonging to a subclass in which the requirement for Mg\(^{2+}\) and any response to calmodulin are difficult to demonstrate.

There are two known mechanisms by which the plasma membrane of a cell keeps the intracellular free calcium concentration low (about 0.1 \(\mu\)M) as compared to extracellular concentration of more than 1 mM. One of these mechanisms employs an ATP-utilizing calcium pump, which involves splitting of ATP by Ca\(^{2+}\)-Mg\(^{2+}\) ATPase. This calcium pump is well characterized in the case of red blood cell plasma membranes (1, 2), where Ca\(^{2+}\)-Mg\(^{2+}\) ATPase has been purified to homogeneity (3) and reconstituted into functionally active vesicles (4). Similar high affinity Ca\(^{2+}\)-Mg\(^{2+}\) ATPase have also been identified in plasma membranes from brain (5, 6), adipocytes (7), pancreatic islets (8), and sperm (9). By analogy to erythrocytes, these enzymes have been suggested to be responsible for a calcium pump in the above mentioned tissues. Another mechanism utilized by the cell to pump calcium out of the cell is via Ca:Na exchange (10, 11) as described in the case of excitable tissue.

In the female reproductive system, no concrete information is available on the role of calcium during the various metabolic events, and it is not known whether a high affinity Ca\(^{2+}\)-Mg\(^{2+}\) ATPase is present in female ovarian tissue.

In a series of papers, Bramley and Ryan (12-15) have described the properties of surface membrane fractions, isolated from the rat ovaries at various stages of the reproductive cycle, as modified by injecting the appropriate hormones. For example, fractionation of granulosa cells yields a plasma membrane fraction (d. 1.16 to 1.18) which is enriched in Mg\(^{2+}\)-dependent ATPase, 5'-nucleotidase, and much of the adenylate cyclase of the ovary. The adenylyl cyclase activity of these membranes was always accompanied by a low level of gonadotropin binding activity. Luteinization of the follicle, following a hCG injection, results in a marked increase in the microvillous region of the luteal cells. When heavily luteinized ovaries are fractionated, two surface membrane fractions can be isolated. A light membrane fraction (membrane from microvillous surface, d. 1.12 to 1.13), which consists of small vesicles, is enriched in hCG binding, 5'-nucleotidase, Mg\(^{2+}\)-dependent ATPase, and (Na + K)-ATPase activities, but possesses little adenylyl cyclase. In contrast, the heavy membrane fraction (basolateral surface membrane, d. 1.16 to 1.18), which consists of large membrane sheets with junctional complexes, is enriched in the basal, hCG, and fluoride-stimulated adenylyl cyclase, but not enriched in other plasma membrane markers. Thus, heavy membranes contain both the adenylyl cyclase as well as a low level of gonadotropin binding activity, while light membranes have considerable hCG binding activity with little adenylyl cyclase.

In this paper, we report the presence and some properties of a high affinity Ca\(^{2+}\)-stimulated and Mg\(^{2+}\)-dependent ATPase in the two plasma membrane fractions isolated from rat corpus luteum.*
**MATERIALS AND METHODS**

**Animals**—Young, immature female Holtzman rats, 22 to 25 days old and weighing between 90 and 100 g were obtained from the Holtzman Company, Madison, WI. **Hormones**—Pregnant mare serum gonadotropin was obtained from the Sigma Chemical Company, St. Louis, MO. Human chorionic gonadotropin was purchased from Ayrest, New York, NY.

**Chemicals**—Carrier-free [γ-32P]ATP was obtained from New England Nuclear, Boston, MA. Chemicals used in enzymic assays were obtained from the Sigma Chemical Company, St. Louis, MO. All other chemicals were of reagent grade.

**Treatment of Animals**—Immature, 22- to 25-day-old female Holtzmann rats, weighing 100 g, were injected with 1 IU of PMSG 0.9 ml of 0.9% NaCl, followed 63 h later with 50 IU of hCG, also in 0.5 ml of 0.9% NaCl. Rats were sacrificed by cervical dislocation on the eighth day after hCG injection, and ovaries were quickly removed. Injection of hCG, after priming with PMSG, resulted in extensive luteinization of ovaries as described before (12, 13).

**Isolation of Light and Heavy Plasma Membranes from PMSG and HCG Primed Ovaries**—Luteinized ovaries were fractionated essentially as described before (13, 14). Eight days after the injection of hCG, rats were sacrificed by cervical dislocation, ovaries were removed, and freed of fat and connective tissues, blotted dry, weighed, minced and homogenized in 20 volumes of 0.1 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, using eight complete strokes of a loose Dounce homogenizer. EDTA was omitted in the first centrifugation, sediment was gently suspended in 0.3 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. The resuspended pellet and the supernatant were then centrifuged at the same speed and for the same time again for maximal separation of each membrane fraction. A systematic outline of this procedure is described in Reference 15. After each primary centrifugation, sediment was gently suspended in 0.3 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, and stored in different aliquots in a liquid nitrogen bath.

**Density Gradient Centrifugation**—All the sucrose solutions were prepared in 10 mM Tris-HCl buffer, pH 7.4, with or without 1 mM EDTA and their concentration adjusted exactly prior to use with an Abbe refractometer.

**Heavy Membranes**—The 800 × g pellet suspended in 20% sucrose was layered on the top of a discontinuous sucrose gradient containing 5 ml of 30%, 8 ml of 36%, 8 ml of 40%, and 5 ml of 50% sucrose solutions. The gradients were centrifuged at 63,000 × g average for 60 min in a Beckman SW27 rotor. Materials accumulating at the interface between 30% and 36% sucrose were collected, diluted four times with 40 mM Tris-HCl buffer, pH 7.4, and sedimented at 10,000 × g average for 10 min. The final pellet was gently resuspended thoroughly in 10 mM Tris-HCl buffer, pH 7.4, and stored in different aliquots in a liquid nitrogen bath.

**Light Membranes**—The 20,000 × g pellet suspended in 20% sucrose was layered on the top of a continuous gradient, prepared using 12 ml of 20% and 12 ml of 55% sucrose solutions, according to the method of Stone (16). After the centrifugation at 63,000 × g average for 4 h in a SW27 rotor, the gradient was fractionated from the top of the tube using a Buchler-Searle auto-densi flow gradient fractionator equipped with a meniscus-sensitive probe. Sucrose concentrations were monitored by a refractometer, and fractions corresponding to 27 to 32% sucrose were pooled, diluted four times with 40 mM Tris-HCl buffer, pH 7.4, and pelleted at 100,000 × g average for 30 min. The final pellet was gently resuspended thoroughly in 10 mM Tris-HCl, pH 7.4, and stored in different aliquots in a liquid nitrogen bath.

**Ca++ ATPase Assay**—The Ca++ ATPase activity was measured by monitoring P in the presence of 36 mM CaCl2, 50 mM Tris-HCl buffer, pH 7.4, 2 mM EDTA-TEA, 0.1 mM ouabain, 5 mM MgCl2, and 5 mM [γ-32P]ATP. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.4, 2 mM EDTA, 0.1 mM ouabain, 5 mM MgCl2, and 5 mM [γ-32P]ATP. The reaction mixture was kept at 37°C for 30 min, and the inorganic phosphate was determined by subtracting the value in the absence of calcium from that in its presence. Incubations were done at 37°C for 30 min periods, and glutamic acid was determined by extracting the organic phase with methanol. The final product was analyzed by extraction of phosphomolybdate complex into the organic phase by the method described by Shami and Radde (18) for placental membranes. A reagent mixture of 0.5 ml contained 20 mM Tris-Cl, pH 8.4, 0.1 mmol NaCl, 0.1 mM MgCl2 or CaCl2, free Mg++, to 5.02 mmol free Ca++, 5 mM 32P and free Mg++, 3.0 mM, 5 mM [γ-32P]ATP (0.1 nCi/nmol), and membrane proteins, 20 to 80 μg. Incubations were done at 37°C for 30 min periods. The procedure to monitor the release of 32P was the same as that used in the assay of high affinity enzyme. Divalent cation-stimulated activity was obtained by subtracting blanks (no protein) and was expressed as nanomoles of ATP split per min per mg of protein used.

**Other Enzymes**—5'-Nucleotidase was measured by the method described by Bramley and Ryan (12), except released inorganic phosphate was monitored by extraction into the organic phase followed by the colorimetric assay using stannous chloride as the reducing agent (18).

**Washing of Membranes with Triton X-100 and/or EDTA**—Two different washing procedures were used for this purpose. In one, membranes were washed in the cold (4°C) with 1 mM EGTA, followed by 0.1 mM EDTA and at last with buffer (10 mM TES-TEA, pH 7.4) alone. In the second procedure, solutions employed in order of suc- cessive washings were 0.02% (w/v) Triton X-100, 1 mM EDTA and buffer (as above). Membranes were centrifuged at 100,000 × g average for 20 min after each washing.

**Calmodulin Assay**—Calmodulin contents of membranes were measured by the method of Wang and Desai (19). This method is based on the ability of calmodulin to stimulate the activity of calmodulin-requiring phosphodiesterase. Phosphodiesterase activity was measured by dephosphorylation of the 5'-AMP produced by the nucleo- tidase. The inorganic phosphate released was determined by the method described in the ATPase assay. The membrane fractions for calmodulin determination were heated in a boiling water bath for 3 min, immediately transferred to ice, and the precipitated protein was removed by centrifugation at 100,000 × g average for 15 min. The supernatant containing released calmodulin was used in the phosphodiesterase assay. The reaction mixture contained 40 mM Tris-HCl, pH 7.5, 40 mM imidazole, 3 mM magnesium acetate, 0.1 mM calcium chloride, 1.2 mM 3',5'-cyclic AMP, 0.2 unit of 5'-nucleotidase, and 20 μg of calmodulin requiring phosphodiesterase in a total volume of 0.5 ml.

**Protein** was determined by the method of Lowry et al. (20), using bovine serum albumin as a standard. Free calcium ion concentrations were calculated by means of a computer program (21) which took into account all complexes involving magnesium, calcium, EGTA, Ca++-ATPase. The calcium ion-sensitive electrode was standardized against optimally buffered solutions of EDTA and calcium chloride in which Ca++ was varied by setting the pH at different values.

**RESULTS**

**Identification of the Calcium-dependent ATPase Activity**

In the initial experiments, where 6 mM MgCl2 was used, an increase in activity on adding Ca++ was not detected because of the high level of low specificity Ca++ or Mg++ ATPase activity present in these membrane fractions (12). A Ca++ or Mg++ ATPase activity was evident, when low or no MgCl2 was employed in the assay. From the results shown in Fig. 2, two kinds of calcium-dependent ATPase activities were detected. One had a high affinity for calcium (see inset also, free calcium ion concentration of half-maximum activity in the range of 0.2 to 0.3 μM) but lower Vmax (nmol min⁻¹ mg⁻¹ protein; light plasma membranes 110, heavy plasma membranes 55). The second type of calcium-dependent ATP splitting activity had lower affinity for calcium (free calcium ion concentration for half-maximum activity in the vicinity of 20 μM) but with a higher Vmax (nmol min⁻¹ mg⁻¹ protein; light
plasma membranes (430), heavy plasma membranes (230).

There was no significant difference in Ca\(^{2+}\)-dependent ATPase activity or its profile (specific activity versus calcium concentration) if 50 \(\mu\)M MgCl\(_2\) (total) was included in the reaction mixture for the ATPase assay. Even in the presence of 50 \(\mu\)M total Mg\(^{2+}\), the free Mg\(^{2+}\) concentration was zero over most of the range of Ca\(^{2+}\) concentrations shown in Fig. 1, except in the last part of the low affinity curve where it did not exceed 4 \(\mu\)M. Note that the free Mg\(^{2+}\) concentration we are talking about here is calculated presuming that the Mg\(^{2+}\)-content of membranes is zero. Later we will show that this apparent lack of any Mg\(^{2+}\) requirement occurs because enough Mg\(^{2+}\) is present in these plasma membranes to satisfy the requirement of high affinity Ca\(^{2+}\)-dependent ATPase.

**Low Affinity Divalent Metal Ion (Ca\(^{2+}\) or Mg\(^{2+}\)) Dependent ATPase**

Fig. 2 shows that calcium and magnesium were equally effective in stimulating the low affinity ATPase in the rat corpus luteum plasma membranes. Here also the \(V_{\text{max}}\) value for the light membranes was higher than that of the heavy membranes. The Ca\(^{2+}\) concentrations required for half-maximum activity were about 1.4 and 1.0 mM (total) for light and heavy membrane fractions, respectively. Magnesium appeared to be slightly more effective than calcium. The concentrations of Mg\(^{2+}\) required for half-maximum activity were 1.0 and 0.8 mM (total) for light and heavy membrane fractions, respectively. In the membrane fractions, the presence of this Ca\(^{2+}\) or Mg\(^{2+}\)-dependent ATPase activity made the quantification of high affinity Ca\(^{2+}\)-ATPase data more difficult. No further attempts were made to characterize this low affinity enzyme.

**Subcellular Localization of the High Affinity Ca\(^{2+}\).Mg\(^{2+}\) ATPase**

The fractionation procedure to isolate light and heavy plasma membranes used here has been published previously (12, 13), and their enzymatic characterization has been done in detail (12-14). From the above studies it was found that the marker enzymes for light membranes are 5'-nucleotidase, Na-K ATPase, and \(^{125}\)I-hCG binding activity while heavy membranes are not rich in the above markers. The adenylate cyclase activity was found to be enriched in the heavy membranes but not in the light membranes. Thus, the heavy membrane fraction can be classified as a surface membrane fraction primarily on the basis of its adenylate cyclase enrichment over the homogenate. This fraction also contains a low level of gonadotropin receptor activity, which can stimulate adenylate cyclase in the presence of hCG (13, 14). In Table I data are shown to compare the distribution of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and 5'-nucleotidase during the fractionation of the PMSG and hCG primed rat ovarian homogenate. The percent recoveries of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and 5'-nucleotidase in the heavy membranes were found to be 4.69 and 4.01, respectively, from the whole homogenate, and enrichment of specific activities over that of homogenate was 1.65- and 1.38-fold, respectively. In the light membrane, Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and 5'-nucleotidase were purified 4.34- and 4.52-fold, respectively, when compared to whole homogenate. The percent recoveries of the above enzymes in this fraction were 5.35 and 5.56, respectively. Thus, the two enzymes were purified to the same extent in the light membrane fraction, while neither was purified significantly in the heavy membrane fraction. Table II compares the specific activities and fold purifications of various enzymes of two membrane fractions.

**Mitochondrial and SER Enzyme Activities in the Plasma Membrane Fractions**

**Mitochondria**—NADH cytochrome c reductase has been used as a mitochondrial marker by Branley and Ryan in the rat luteal subcellular fractions (12). Based on the marker enzyme analysis for the major subcellular organelles and electron microscopic examination of the fractions, these authors have predicted that the mitochondrial contaminations of light and heavy membranes are in the range of 1 to 2% and 8 to 16%, respectively.\(^{1}\)

**SER**—Smooth endoplasmic reticulum is the other subcellular organelle which could contribute, if present, to the Ca\(^{2+}\)-ATPase of the light and heavy membrane fractions. NADPH cytochrome c reductase if assayed in the presence of 0.1% Triton X-100 can be taken as a marker of SER in rat ovarian tissue (12). Again based on the content of this enzyme and

\(^{1}\) T. A. Branley and R. J. Ryan, personal communication.
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Comparison of Ca$^{2+}$-Mg$^{2+}$ ATPase and 5'-nucleotidase distribution in subcellular fractions of rat corpus luteum homogenate

The data shown are mean ± ½ range of two membrane preparations. Each sample is assayed for enzymic activity in triplicate. Enzyme assays are done as described under "Materials and Methods." Free Ca$^{2+}$ concentration used in ATPase assay is 0.29 μM. This relatively low [Ca$^{2+}$] was chosen to avoid any contribution of low Ca$^{2+}$ affinity ATPase to the assay, but it gave an activity of only about half the maximal activity of the high Ca$^{2+}$ affinity ATPase.

| Fraction                  | Ca$^{2+}$-Mg$^{2+}$ ATPase | 5'-Nucleotidase |
|---------------------------|----------------------------|-----------------|
|                           | Specific activity          | Fold purification over homogenate | % Recovery from homogenate | Specific activity | Fold purification over homogenate | % Recovery from homogenate |
| Homogenate                | 8.9 ± 0.25                 | 1.0             | 100                          | 0.1 ± 0.01        | 1.0                          | 100                          |
| 800 × g sediment          | 12.6 ± 0.7                 | 1.4 ± 0.1       | 34.1 ± 1.9                   | 0.18 ± 0.01       | 1.4 ± 0.0                   | 26.1 ± 1.3                   |
| Heavy plasma membrane     | 14.6 ± 1.2                 | 1.6 ± 0.6       | 4.7 ± 0.4                    | 0.22 ± 0.02       | 1.6 ± 0.1                   | 4.0 ± 0.8                    |
| 20,000 × g sediment       | 15.6 ± 0.5                 | 1.7 ± 0.0       | 28.6 ± 3.1                   | 0.25 ± 0.02       | 1.7 ± 0.1                   | 27.3 ± 5.5                   |
| Light plasma membrane     | 38.8 ± 2.0                 | 4.3 ± 0.3       | 5.3 ± 1.1                    | 0.76 ± 0.27       | 4.5 ± 0.3                   | 5.6 ± 1.0                    |

Table II

Marker enzymes of light and heavy plasma membranes

The data shown for Ca$^{2+}$-Mg$^{2+}$ ATPase and 5'-nucleotidase are mean ± ½ range of two membrane preparations. Each fraction is assayed for its enzymic activity in triplicate. Data for Na-K ATPase, hCG binding, and adenyl cyclase activity are unpublished data provided by T. A. Bramley and R. J. Ryan. Ca$^{2+}$-Mg$^{2+}$ ATPase is measured at free Ca$^{2+}$ concentration of 0.29 μM.

| Enzyme          | Light plasma membrane | Heavy plasma membrane |
|-----------------|-----------------------|-----------------------|
|                 | Specific activity     | Fold purification over homogenate | Specific activity | Fold purification over homogenate | % Recovery from homogenate |
| Ca$^{2+}$-Mg$^{2+}$ ATPase | 38.8 ± 2.0           | 4.3                   | 14.8 ± 1.2           | 1.6                   |
| 5'-Nucleotidase | 0.76 ± 0.01           | 4.5                   | 0.23 ± 0.01          | 1.4                   |
| Na-K ATPase     | 15.8 ± 1.5            | 9.2                   | 4.8 ± 0.7            | 2.2                   |
| hCG binding     | 5.7 ± 0.5             | 9.5                   | 1.6 ± 0.3            | 2.5                   |
| Adenyl cyclase  | 0.06 ± 0.01           | 0.4                   | 0.56 ± 0.01          | 4.3                   |
| Basal           | 0.03 ± 0.02           | 0.2                   | 1.40 ± 0.10          | 4.3                   |
| hCG stimulated  | 0.44 ± 0.13           | 0.3                   | 9.59 ± 0.63          | 6.7                   |
| NaF stimulated  |                       |                       |                      |                      |

"nmol min"$^{-1}$ mg$^{-1}$.
"μmol min"$^{-1}$ mg$^{-1}$.
"μmol h"$^{-1}$ mg$^{-1}$.
"nmol of 125I-hCG bound per mg$^{-1}$.
"nmol h"$^{-1}$ mg$^{-1}$.

Magnumes Requirements of the High Affinity Ca$^{2+}$ ATPase

The results indicate (Fig. 1) that the addition of external magnesium (50 μM total) does not affect the Ca$^{2+}$-Mg$^{2+}$ ATPase activity appreciably. The high affinity Ca$^{2+}$-Mg$^{2+}$ ATPases in the red blood cell (3, 4) and the brain (5) require magnesium for the expression of their activity. In the case of Ca$^{2+}$ ATPase of the rat corpus luteum membranes, there are two possibilities. Either this enzyme does not require magnesium or the magnesium content of the membrane (isolated, even in the presence of 1 mM EDTA) is enough to satisfy the Mg$^{2+}$ requirement for the expression of the enzyme activity. To test the above possibilities, the enzyme was assayed using the Ca-EGTA and Ca-CDTA systems. EGTA chelates Ca$^{2+}$ more effectively than Mg$^{2+}$ while CDTA is equally effective in chelating both the divalent cations. Thus, when EGTA is present in the reaction mixture free Mg$^{2+}$ is available. In contrast when CDTA is used all the Mg$^{2+}$ can be completely complexed. Fig. 3 shows the results of an experiment in which the specific activities of Ca$^{2+}$-Mg$^{2+}$ ATPase were determined at various concentrations of free Ca$^{2+}$, using the Ca-EGTA and Ca-CDTA systems. The specific activity of Ca$^{2+}$-Mg$^{2+}$ ATPase was considerably reduced when free Mg$^{2+}$ was chelated by CDTA. At concentrations of free Ca$^{2+}$ higher than 2 μM, the enzyme activity approached similar values, whether the free Mg$^{2+}$ was available or complexed. This was because at such calcium concentrations, the low affinity Ca$^{2+}$ or Mg$^{2+}$ ATPase was also expressed.

Another proof that Mg$^{2+}$ is required for the activity of high affinity calcium-stimulated ATPase came from the experiment in which the amount of free Mg$^{2+}$ was increased under the conditions when either the amount of free Ca$^{2+}$ present in the system was zero or was fixed to 0.56 μM. To achieve this, two systems were used, Ca-Mg-CDTA where free Ca$^{2+}$ was kept constant at 0.56 μM and Mg-CDTA, where all the Ca$^{2+}$ was complexed. In both of the above systems, free Mg$^{2+}$ was increased and the specific activity of the enzyme measured. When the free Mg$^{2+}$ was increased from zero to 200 μM and the free Ca$^{2+}$ was kept at zero, light membranes did not show any ATPase activity and the heavy membranes showed minimal activity (Fig. 4). Even when free Ca$^{2+}$ was 0.56 μM and free Mg$^{2+}$ was less than 100 μM, no significant activity was detected in either fraction. Only at Mg$^{2+}$ concentrations above 100 μM was a significant activity evident. No attempt was made to find the amount of Mg$^{2+}$ required to saturate the enzyme. The experiment proves clearly that Mg$^{2+}$ is required for the enzyme activity, but Mg$^{2+}$ alone is not enough. In the
Fig. 4. Dependence of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase on free Mg\(^{2+}\) but Mg\(^{2+}\) alone is not enough, free Ca\(^{2+}\) is also required. Left curve represents the data on light membranes and curve on right is for heavy membranes. △, ○ show activity using Ca-Mg-CDTA system where free Ca\(^{2+}\) concentration is constant (0.56 μM). △, ○, represents the enzyme activity when free Ca\(^{2+}\) concentration is zero. Mg\(^{2+}\) concentration shown for abscissa is calculated using a computer program.

**Table III**

**Effect of various agents on Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity**

The data shown are mean ± S.D. of enzyme activity assayed in triplicate. Ca\(^{2+}\)-Mg\(^{2+}\) ATPase is assayed under standard conditions as described under "Materials and Methods" at free Ca\(^{2+}\) concentrations of 0.29 μM.

| Addition or subtraction | Specific activity (per cent of control) |
|-------------------------|----------------------------------------|
|                         | Light plasma membrane | Heavy plasma membrane |
| Complete                | 100 ± 3.4              | 100 ± 14.8             |
| +20 mM KCl             | 95.7 ± 8.7             | 100.7 ± 9.0            |
| +20 mM NaCl            | 96.8 ± 6.8             | 102.8 ± 16.6           |
| -0.1 mM ouabain        | 104.3 ± 9.4            | 115.8 ± 10.0           |
| +20 mM NaN\(_2\)       | 97.6 ± 9.1             | 99.5 ± 9.9             |
| +0.2 mM oligomycin     | 116.7 ± 15.1           | 87.8 ± 3.4             |
| +6.1 mM DCCD           | 89.1 ± 4.7             | 101.3 ± 5.9            |

Ca-Mg-CDTA system, free Ca\(^{2+}\) concentration was kept constant using a Ca\(^{2+}\)-sensitive electrode.

**Effect of Various Agents on Ca\(^{2+}\)-Mg\(^{2+}\) ATPase Activity**

The effect of various agents on the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase was tested in order to compare its properties to those of the well-studied and characterized Ca\(^{2+}\)-Mg\(^{2+}\) ATPase of red blood cell ghosts. Including 20 mM KCl, 20 mM NaCl and excluding 0.1 mM ouabain had no significant effect on the enzyme activity observed (Table III). There was no significant inhibition of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase when sodium azide (20 mM), oligomycin (0.2 mM), and DCCD (0.1 mM) were included in the assay mixture individually (Table III). These agents are specific inhibitors of the mitochondrial Ca\(^{2+}\) ATPase.

**Calmodulin Contents of the Membranes**

Table IV compares the calmodulin contents of two membrane fractions from the rat corpus luteum. Both fractions contain approximately equal amounts of calmodulin per mg of protein, values which are not too different from the value observed for red blood cell ghosts from our laboratory. The synaptic plasma membranes and luteolus-extracted microsomes from brain contain several more calmodulin than the corpus luteum rat membrane fractions. However, treatment of these membranes with a low concentration of Triton X-100 and/or EGTA did not result in the drastic decrease in the calmodulin contents (Table V) which has been observed with red blood cell ghosts and brain microsomes. Although treatment of heavy membranes with 0.02% Triton X-100 followed by 1 mM EGTA and washing reduced the calmodulin contents by 50% we failed to see a stimulation of Ca\(^{2+}\) ATPase activity by externally added calmodulin.

**Effect of Calmodulin on Ca\(^{2+}\) ATPase**

Table V shows the results of an experiment in which calmodulin was added in the Ca\(^{2+}\) ATPase assay reaction mixture and enzyme activity was measured. Statistical analysis of the results shows that calmodulin stimulation of Ca\(^{2+}\) ATPase is not significant (p > 0.05). The experiment shown is typical and in the majority of experiments, where increasing amounts of calmodulin were added (up to 11 μg) at the various fixed concentrations of free Ca\(^{2+}\) (up to 1.73 μM), no significant stimulation of the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase was observed. This was true of plasma membranes isolated in the presence of 1 mM EGTA and those preparations where membrane fractions are washed with Triton X-100 and/or EGTA after preparation as in Table IV.

**Effect of Phenothiazine Drugs on Ca\(^{2+}\) ATPase**

Phenothiazine drugs have been shown to inhibit the calmodulin stimulation of phosphodiesterase (22) and high affinity Ca\(^{2+}\) ATPase (23). This effect is thought to be because of the binding of these drugs to calmodulin. Since calmodulin in the rat luteal plasma membranes is not easily dissociated, we studied the effect of phenothiazine drugs (trifluoperazine and chlorpromazine) on the activity of Ca\(^{2+}\) ATPase in a further attempt to demonstrate its calmodulin sensitivity. Trifluoperazine (see Table VI) up to a concentration of 50 μM caused no

**Table IV**

Calmodulin contents of rat luteal plasma membranes

The data shown are mean ± ½ range of two membrane preparations. Calmodulin contents were quantitated by measuring the activation of calmodulin-free phosphodiesterase, as described under "Materials and Methods." Each assay was done in triplicate.

| Fraction | Calmodulin |
|----------|------------|
| Light plasma membrane | Heavy plasma membrane |
| 211 ± 13 | 216 ± 0 |
| 194 ± 0 | 184 ± 4.6 |
| 198 ± 3.2 | 110 ± 1.0 |

**Table V**

Effect of calmodulin on Ca\(^{2+}\)-Mg\(^{2+}\) ATPase of plasma membranes fractionated without and with 1 mM EGTA

Data are mean ± S.D. (N = 6). Plasma membranes were isolated with and without EGTA present in homogenization buffer and sucrose solutions and checked for Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity the same day. EGTA in parenthesis indicates membranes were isolated in the presence of 1 mM EGTA. Assay conditions were the same as described under "Materials and Methods" except: 20 μM MgCl\(_2\) (total), 20 mM NaCl, 50 mM KCl were also present.

| Addition | Light plasma membrane | Light plasma membrane (EGTA) | Heavy plasma membrane | Heavy plasma membrane (EGTA) nMol.min\(^{-1}\)-mg\(^{-1}\) |
|----------|-----------------------|-----------------------------|-----------------------|----------------------------------|
| 0.13 μM free Ca\(^{2+}\) alone | 50.8 ± 5.8 | 45.7 ± 4.5 | 21.1 ± 2.9 | 14.0 ± 2.1 |
| 0.13 μM free Ca\(^{2+}\) + 2 μg of calmodulin | 52.6 ± 5.3 | 48.3 ± 4.1 | 24.1 ± 1.7 | 14.5 ± 2.8 |
| Calmodulin stimulation | 1.8 ± 7.8 | 2.6 ± 6.1 | 3.0 ± 3.4 | 0.5 ± 3.5 |
| % stimulation | 3.5 | 5.7 | 14.7 | 3.6 |
| P | 0.29 | 0.32 | 0.05 | 0.73 |
**TABLE VI**
Effect of trifluoperazine on Ca\(^{2+}\)-Mg\(^{2+}\) ATPase of light and heavy plasma membranes

Data shown are mean ± S.D. of assays done in triplicate. Controls were run to check the effect of trifluoperazine on extraction of \(^{32}P\) released by isobutanol/benzene as used in ATPase assay. Free Ca\(^{2+}\) concentration used is 1.8 µM.

| Trifluoperazine (µM) | Percentage of specific activity Light plasma membrane | Heavy plasma membrane |
|----------------------|------------------------------------------------------|-----------------------|
| 0                    | 100 ± 4.3                                            | 100 ± 1.9             |
| 10                   | 115.0 ± 3.7                                         | 98.4 ± 0.6            |
| 20                   | 106.1 ± 8.6                                         | 96.1 ± 0.8            |
| 50                   | 104.9 ± 1.6                                         | 96.4 ± 0.6            |
| 100                  | 94.4 ± 0.5                                          | 85.2 ± 0.3            |

**TABLE VII**
Effect of chlorpromazine and calmodulin on Ca\(^{2+}\)-Mg\(^{2+}\) ATPase of plasma membranes

Data shown are mean ± S.D. of assays done in duplicate. Controls were run to check the effect of chlorpromazine on the extraction of \(^{32}P\).

| Addition | Percentage of specific activity Light plasma membrane | Heavy plasma membrane |
|----------|------------------------------------------------------|-----------------------|
| 1.8 µM free Ca\(^{2+}\) | 100 ± 5.7                                           | 100 ± 12.5            |
| 1.8 µM free Ca\(^{2+}\) + 500 µM chlorpromazine | 75.9 ± 5.8                                           | 96.3 ± 10.2           |
| 1.8 µM free Ca\(^{2+}\) + 500 µM chlorpromazine + 50 µg of calmodulin | 80.3 ± 5.8                                           | 78.7 ± 8.4            |

significant inhibition of the Ca\(^{2+}\) ATPase activity in the light and heavy plasma membranes. Table VII shows the results when 500 µM chlorpromazine was used in the assay mixture.

Inhibition of Ca\(^{2+}\) ATPase of light plasma membrane even with this high concentration of chlorpromazine is only in the vicinity of 24%. Addition of 50 µg of calmodulin could not restore the activity of the chlorpromazine-inhibited enzyme to the original levels as shown in the data in Table VII.

**DISCUSSION**

The data indicate the presence of the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase with a high affinity for calcium in the two plasma membrane fractions isolated from the rat corpus luteum. In the light membrane, 5'-nucleotidase and Ca\(^{2+}\)-Mg\(^{2+}\) ATPase copurify to the extent of 4.5- and 4.3-fold, respectively, over the homogenate, suggesting that the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase studied in this fraction is of plasma membrane origin. In the heavy membrane fractions, neither 5'-nucleotidase nor Ca\(^{2+}\)-Mg\(^{2+}\) ATPase were enriched significantly when compared to the homogenate (Table I).

When granulosa cells (the precursors of luteal cells) are fractionated, a surface membrane fraction accumulates in a density gradient region of 1.15 to 1.18 g/cm\(^3\) (15). This fraction is enriched in Mg\(^{2+}\)-dependent ATPase, 5'-nucleotidase, and much of the adenylate cyclase of the ovary (15). Luteinization of the follicle following a hCG injection causes differentiation, with the appearance of luteal cells, containing a large microvillous region. When luteinized ovaries are subjected to fractionation, an additional surface membrane can be isolated at a density region of 1.12 to 1.13 g/cm\(^3\) (called light membrane fraction because of its lower buoyant density). Since the light membrane fraction is obtained as a result of extensive luteinization of the ovaries (appearance of the microvillous region) this is presumed to derive from the microvillous region of the luteal cells. The luteinized ovaries also yield a surface membrane fraction at a buoyant density of 1.16 to 1.18 g/cm\(^3\) (heavy membrane) similar to that of the granulosa cells.

Light membranes from the luteinized ovaries are enriched in several plasma membrane markers, but not in adenylate cyclase. Heavy membranes (d. 1.16 to 1.18) from luteinized ovaries are not enriched in most plasma membrane markers but are enriched in adenylate cyclase. Since luteal cells are transformed granulosa cells it is unlikely that the granulosa cell plasma membranes (d. 1.16 to 1.18) will completely disappear as a result of luteinization of the follicles. Therefore, though the heavy membrane fraction from the luteal cells lacks enrichment of the surface membrane markers, it has been suggested to be a plasma membrane fraction from the basolateral surface of the luteal cells.

This inference is further supported by the fact that digitonin had differential effects on the light and heavy membranes (24). In the above study (24) it was found that the buoyant density of luteal cell light membrane as marked by \(^{35}S\)hCG binding, Mg\(^{2+}\) ATPase, and 5'-nucleotidase was highly perturbable by digitonin (5 density > 0.05). Therefore, if the low level of 5'-nucleotidase activity found in the heavy membranes is because of the contamination of this fraction by light membranes, one would expect it to be as perturbable as the light membrane 5'-nucleotidase. However, it was found that the buoyant density of luteal cell heavy membrane fraction, as marked by adenylate cyclase, Mg\(^{2+}\) ATPase, and 5'-nucleotidase was not significantly perturbed by digitonin (24). Thus, it is reasonable to infer that the low level of 5'-nucleotidase detected in the heavy membrane truly belongs to this subcellular fraction. Similarly, it may also be possible that the low level of Ca\(^{2+}\) ATPase in heavy membranes (although not enriched over homogenate) is truly a part of the basolateral surface of rat luteal cells.

The calcium concentration for half-maximal activity of the high affinity ATPase is in the range of 0.25 µM, making it possible for it to be of physiological significance in removing intracellular Ca\(^{2+}\).

A low affinity Ca\(^{2+}\) or Mg\(^{2+}\) ATPase is also present in these membrane fractions (Figs. 1 and 2). Such a low affinity divalent metal ion (Ca\(^{2+}\) or Mg\(^{2+}\)) ATPase has been shown to be present in various tissues including placenta (18), kidney (25), liver (26), and intestine (27). The presence of such a low affinity enzyme made the quantitation of the high affinity Ca\(^{2+}\)-Mg\(^{2+}\) ATPase difficult. It was possible to measure the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase with a high affinity for calcium only when a zero or low concentration of magnesium was employed in the reaction mixture.

The low contamination of smooth endoplasmic reticulum and mitochondrial enzymes suggests that the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase we are studying comes from plasma membrane. The effect of inhibitors of the mitochondrial ATPase, sodium azide, oligomycin, and DCCD were studied to further check mitochondrial contamination of the plasma membranes obtained. In fact, no significant effect of these compounds was found, and this indicated that the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase studied here was not because of mitochondrial contamination in the plasma membranes. The high affinity Ca\(^{2+}\)-Mg\(^{2+}\) ATPase from the endoplasmic reticulum of adipocytes has a requirement for Mg\(^{2+}\) and K\(^{+}\) (28). KCl (20 mM) has no effect on the rat corpus luteum Ca\(^{2+}\)-Mg\(^{2+}\) ATPase suggesting that it is different from the adipocyte endoplasmic reticulum enzyme. Although from the data on the marker enzyme of SER it is not possible to absolutely rule out that the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase studied here could not be of the SER origin, yet copurification of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase and 5'-nucleotidase suggests that enzyme is present in the plasma membranes.

The enzyme in two rat corpus luteum membranes is present at a considerably higher specific activity than the red cell.
High Affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase in Rat Corpus Luteum Plasma Membranes

ghost and has a \( V_{\text{max}} \) of 110 and 55 nmol min\(^{-1}\) mg\(^{-1}\), respectively, for the light and the heavy membranes, as compared to 20 nmol min\(^{-1}\) mg\(^{-1}\) for the red blood cell ghost.\(^\text{3}\) All three membrane preparations show similar affinity for calcium (Ca\textsuperscript{2+} in the range of 0.2 to 0.3 \( \mu \)M).

The rat corpus luteum enzyme is different from the red blood cell ghosts enzyme in the respect that enough magnesium is present in the corpus luteum plasma membranes to satisfy the magnesium requirement from the enzyme. Special conditions were needed, like chelation of magnesium with CDTA, to show the requirement for magnesium. CDTA has an equal affinity for calcium and the magnesium while EGTA has a greater affinity for calcium than magnesium. Therefore, it was possible to use a low concentration (2 mM) of CDTA to control the free Ca\textsuperscript{2+} while keeping free magnesium very low. From the experiments in Figs. 2 and 3, we confirmed that Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase from the rat corpus luteum requires Mg\textsuperscript{2+} for activity and that Mg\textsuperscript{2+} alone is not enough for the expression of the activity. By analogy to the red blood cell enzyme, Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase of the rat corpus luteum may be responsible for a calcium-pumping mechanism in these cells. The corpus luteum enzyme was found to be very similar to the properties of the adipocyte plasma membrane Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase (7) which shows a similar type of magnesium requirement.

Light and heavy plasma membranes from the corpus luteum were found to contain an appreciable amount of calmodulin (nanograms per mg of protein, light 221 ± 13, heavy 216 ± 0, mean ± 1/2 range of two membrane preparations) (Table V). These contents could not be reduced drastically by washing with the 1 mM EGTA and/or 0.01% Triton X-100. Such washed or unwashed membranes did not show calmodulin stimulation in the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase assay (Table VI). Thus, it appears that calmodulin is more tightly bound to rat luteal cell plasma membranes than is the case with red blood cells or brain synaptic plasma membranes (5). From the experiments where calmodulin and/or phenothiazine drugs were included in the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase assay, we cannot draw any conclusions about the calmodulin sensitivity of the rat corpus luteum Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase, under the conditions of assay and isolation of membrane we employed.

No concrete information is available about the role of calcium in the corpus luteum function and/or reproductive cycle of the female. The calcium content of cumulus-enclosed oocytes of the rat was found to increase after injection of PMSG reaching a maximum 55 h later, when ovulation occurs (29). It is known that corpus luteum secretes progesterone under the influence of luteinizing hormones by activating the membrane-bound adenylylate cyclase. The resulting cyclic AMP is known to stimulate the protein kinase activity (30). No definite role is known for Ca\textsuperscript{2+} in regulation of corpus luteum but in view of the large changes in intracellular Ca\textsuperscript{2+} it is probable that Ca\textsuperscript{2+} is an important intracellular messenger in this system.

We found strong similarities in the properties of Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPases of plasma membranes from the rat corpus luteum (as reported in this paper) and adipocytes (7). In both cases, the same type of special effort was needed to show the magnesium requirement. In adipocyte plasma membranes, any response of the Ca\textsuperscript{2+}-stimulated ATPase to calmodulin has been difficult to demonstrate,\(^\text{3}\) but calmodulin has been shown to stimulate the transport of Ca\textsuperscript{2+} in the plasma membrane vesicles of adipocytes (31). In our system, no stimulatory effect of calmodulin on the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase was found, and the effect of calmodulin on Ca\textsuperscript{2+} transport was not investigated.

Like the Ca\textsuperscript{2+} ATPases from erythrocytes and brain, the corpus luteum and adipocyte enzymes are plasma membrane enzymes with a high affinity for Ca\textsuperscript{2+}, suggesting that all of these enzymes are Ca\textsuperscript{2+} extrusion pumps. However, the differences mentioned above indicate that tissue-specific differences exist which distinguish the adipocyte and luteal enzymes from those of the erythrocyte and brain.

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