Biochimica et Biophysica Acta, 397 (1975) 254—266

BBA 67544

SUBCELLULAR LOCALIZATION AND PARTIAL CHARACTERIZATION OF BOVINE CORPUS LUTEUM ADENYLATE CYCLASE*

KIRPAL S. SIDHU**, TIN Y. LIU, CATHLYNE E. CAMP and JOHN M. MARSH

The Endocrine Laboratory and the Department of Biochemistry, University of Miami School of Medicine, Miami, Fla. 33152 (U.S.A.)

(Received February 3rd, 1975)

Summary

The subcellular localization of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) in bovine corpus luteum was studied using isotonic and hypotonic homogenization and fractionation conditions. All fractions prepared were assayed for adenylate cyclase, marker enzymes and DNA. Only plasma membrane marker enzyme, 5′-nucleotidase paralleled the distribution of adenylate cyclase under both isotonic and hypotonic conditions (coefficient of correlation = 0.95). Two main fractions prepared under hypotonic conditions were subfractionated by discontinuous sucrose gradient centrifugation. The highest amount of adenylate cyclase was found in a fraction having a density approximately equal to 1.13 g/cm^3. The specific activity of this fraction was 4—6 times higher than that of the homogenate. The electron microscopic study of this fraction revealed the presence of a single type of particulate material consisting of small vesicles exhibiting a typical unit membrane structure. It is concluded that this adenylate cyclase is primarily localized in the plasma membranes. Basal adenylate cyclase activity of plasma membranes was stimulated 2—3 times by luteinizing hormone (10 μg/ml), 3—4 times by prostaglandin E_2 (10 μg/ml), 4—6 times by NaF (0.01 M) and two times by methanol (0.2%).

Introduction

The stimulation of steroidogenesis by luteinizing hormone in bovine corpus luteum [1] appears to be mediated by adenosine 3′,5′-monophosphate

* A preliminary report on a part of this research study was presented at the 1974 Meeting of the Federation of American Societies for Experimental Biology held at Atlantic City, New Jersey [40].

**Present address: Department of Public Health, The State of Michigan, 3500 North Logan Street, Lansing, Mich. 48914 (U.S.A.).

[2—5]. In order to understand how luteinizing hormone brings changes in adenosine 3′,5′-monophosphate (cyclic AMP) and steroidogenesis it is necessary to know the site of its action. It is generally presumed that since luteinizing hormone is a protein it does not penetrate the plasma membranes and probably stimulates the corpus luteum adenylate cyclase (EC 4.6.1.1) on the surface of the cell. A plasma membrane fraction from bovine corpora lutea has been shown to contain a hormone-sensitive adenylate cyclase [6]. However, the complete subcellular distribution of this enzyme has not been described. The importance of this question is emphasized by the reports indicating that homogenates of luteinized rat ovaries have more than one sedimentable component with a marked binding affinity for radiolabelled gonadotropins [7,8]. The purpose of this investigation was to thoroughly document the subcellular localization of this adenylate cyclase by correlating its activity in subcellular fractions with classical markers and confirming this localization by electron microscopy.

Materials and Methods

Materials

Bovine luteinizing hormone (NIH-LH-B-7) was supplied by National Institutes of Health, Bethesda, Md. Prostaglandin E_2 was a gift from the Upjohn Co., Kalamazoo, Mich. Adenosine 5-tri[α-^{32}P] phosphate tetrasodium salt, (spec. act. 8.7 Ci/mmol) was obtained from International Chemical and Nuclear Corp., Irvine, Calif. The [8-^{3}H]adenosine 3′,5′-cyclic phosphate ammonium salt, (spec. act. 27.5 Ci/mmol) was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Cyclic AMP, 5′-AMP, ATP, GTP, and Tris were purchased from Sigma Chemical Co., St. Louis, Mo. Theophylline was obtained from Nutritional Biochemicals, Cleveland, Ohio.

Homogenization and fractionation under isotonic conditions

Ovaries from cows in first 6 months of pregnancy as determined by the size of the fetus [9] were obtained at slaughter and transported to the laboratory in ice-cold 154 mM NaCl. The corpora lutea were dissected free of adjacent ovarian tissue in a cold room at 4°C. Each corpus luteum was cut into four sections and the luteal tissue was separated from its connective tissue capsule by using the blunt edge of a Stadie-Riggs slicing blade. The luteal tissue was finely minced with a pair of scissors. Approx. 5 g of minced corpus luteum tissue were homogenized (1 : 3, w/v) in an isotonic homogenizing medium consisting of 0.25 M sucrose, $3 \cdot 10^{-3}$ M $MgCl_2$ and 0.05 M Tris, pH 7.4 (Fig. 1). Homogenization was accomplished with eight up and down strokes of a motor-driven Potter-Elvehjem glass-Teflon homogenizer. The homogenate was then strained through four layers of cheese cloth to remove unhomogenized tissue and connective tissue. The homogenate was fractionated as shown in Fig. 1 by differential centrifugation using a refrigerated Lourdes centrifuge (Model A-2, rotor 9 RSL) and a Beckman ultracentrifuge (Model L, rotor Type 40). The fractions consisted of five pellets and a supernatant. Each pellet was washed once with the homogenizing medium and suspended in an assay medium (0.02 M glycylglycine, 0.01 M $MgSO_4$, pH 7.4) for assays of various

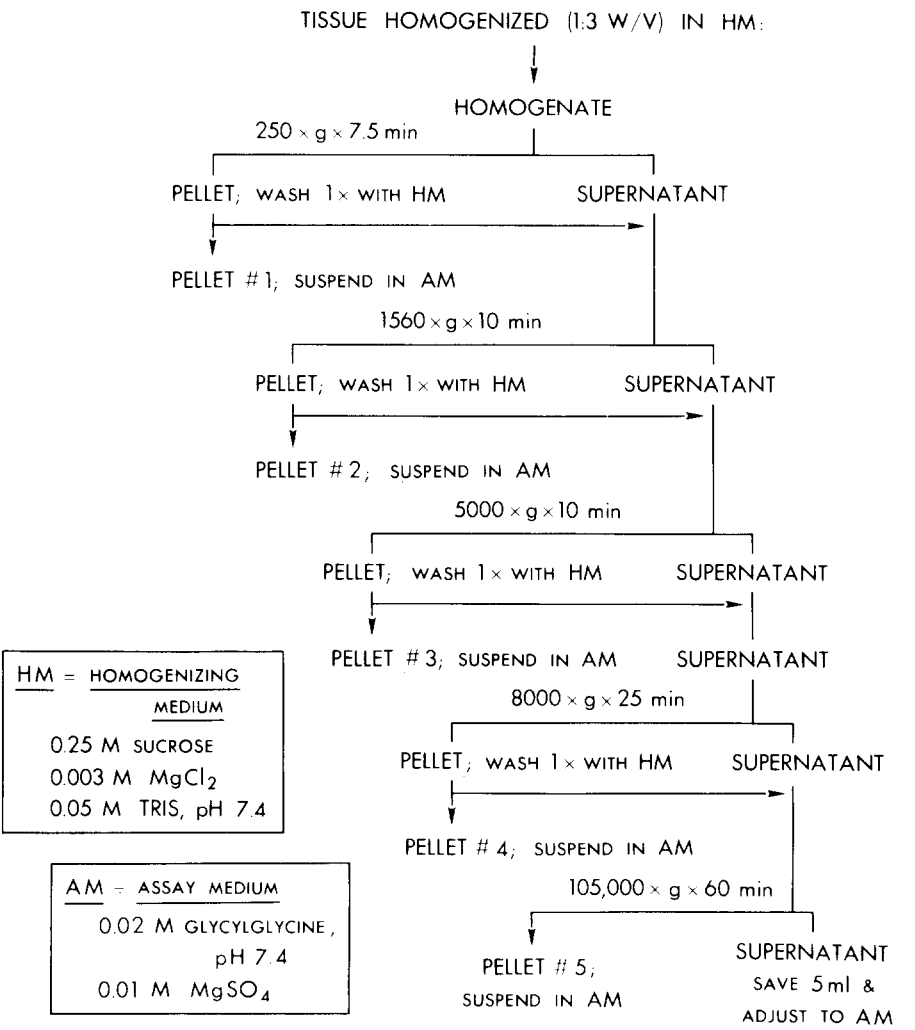

Fig. 1. Homogenization and fractionation of bovine corpora luteal tissue under isotonic conditions. Procedural details are described under Materials and Methods.

enzymes. The supernatant was adjusted with a small volume of 0.2 M glycylglycine, 0.1 M $MgSO_4$, pH 7.4, so that it contained the same concentration of glycylglycine and $MgSO_4$ as in the suspended pellet fractions.

Homogenization and fractionation under hypotonic conditions

The homogenization and fractionation under hypotonic conditions was adapted from the procedure of Neville [10] which is directed toward the isolation of the plasma membranes. About 5 g of minced bovine corpus luteum tissue, prepared as described above, were homogenized (1 : 4, w/v) in a hypotonic homogenizing medium consisting of 1 mM $NaHCO_3$ and 5 mM dithiothreitol, pH 7.5 (Fig. 2). Homogenization was accomplished with three up and down strokes of a motor-driven Potter-Elvehjem glass-Teflon homogenizer fol-

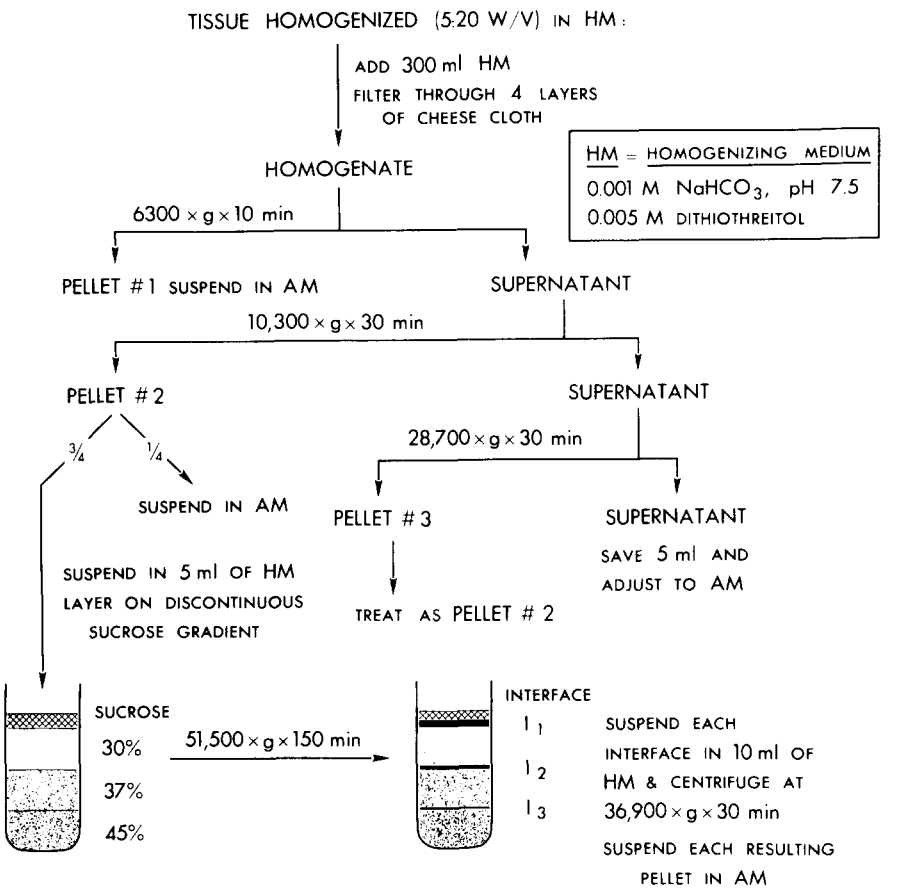

Fig. 2. Homogenization and fractionation of bovine corpora luteal tissue under hypotonic conditions. Procedural details are described under Materials and Methods.

lowed by 20 up and down strokes of a Dounce homogenizer. The homogenate was poured into 300 ml of the ice-cold homogenizing medium, stirred for 2—3 min and strained through four layers of cheese cloth. The strained homogenate was fractionated by differential centrifugation using a refrigerated Lourdes centrifuge (Model A-2, rotor 9 RSL). The fractions consisted of three pellets (pellet 1, 6300 × *g* for 10 min; pellet 2, 10 300 × *g* for 30 min; and pellet 3, 28 700 × *g* for 30 min) and a supernatant (28 700 × *g* for 30 min). In some experiments centrifugation at 10 300 × *g* for 30 min was omitted and only two pellets (6300 × *g* for 10 min and 28 700 × *g* for 30 min) were obtained. In such cases the pellet obtained by centrifugation at 28 700 × *g* for 30 min was designated as P-2+3. Pellet 1 (P-1) and aliquots of pellet 2 (P-2) and pellet 3 (P-3) were suspended in the assay medium (0.02 M glycylglycine and 0.01 M $MgSO_4$, pH 7.4) for the assays of the various enzymes. The supernatant (28 700 × *g* for 30 min) was also adjusted to the assay medium as mentioned previously so that it contained the same concentration of glycylglycine and $MgSO_4$ as in the suspended pellet fractions. Pellet 2, pellet 3, pellet 2+3 were further fractionated by a 3-layered discontinuous sucrose gradient centrifuga-

tion (51 500 × *g* for 150 min; Beckman L ultracentrifuge, spinco SW 25.1 rotor). An aliquot of each pellet was suspended in 5 ml of the homogenizing medium and layered on top of a discontinuous sucrose gradient consisting of (from bottom to top) 6 ml of 45% sucrose (d = 1.20), 8 ml of 37% sucrose (d = 1.16) and 8 ml of 30% sucrose (d = 1.13). This discontinuous sucrose gradient centrifugation fractionated each pellet into three bands at the interfaces of I_1, I_2 and I_3 as shown in Fig. 2. The material from each interface was suspended in 10 ml of the homogenizing medium and centrifuged at 36 900 × *g* for 30 min. The resulting pellet of each interface was suspended in the assay medium for assays of the various enzymes.

Enzymes, DNA and protein assays

Adenylate cyclase was assayed by measuring the conversion of [α-^{32}P] ATP into cyclic [^{32}P] AMP as described by Marsh [3]. Theophyllin (40.0 mM) was included in the reaction mixture to block the phosphodiesterase activity [3]. The final concentration of NaF whenever included was 0.01 M. At the end of the incubation period 62.5 nmol of carrier cyclic AMP was added and the reaction was terminated by immersing the assay tubes in boiling water for 3 min. Cyclic AMP was isolated in accordance with the method of Krishna et al. [11]. The ^{32}P radioactivity in 3 ml supernatant added to 10 ml of scintillation mixture [12] was counted in a scintillation counter. The cyclic [^{32}P] AMP purified by this procedure has been shown to be radiochemically pure after this precipitation step [3]. The procedural losses were corrected by taking into consideration the recovery of carrier cyclic AMP measured by means of its maximal absorption at 257 nM. The enzymatic blank value (0.002% of the total counts) for adenylate cyclase assay was negligible. The addition of theophylline 40.0 mM, in the reaction mixture of this adenylate cyclase assay essentially blocked phosphodiesterase activity for the duration of the assay. At the termination of the reaction, the percentage recovery of cyclic [8-^{3}H] adenosine 3′,5′-monophosphate, included in the reaction mixture of adenylate cyclase assay, was 96.5 ± 1.2 (n = 47).

The effect of luteinizing hormone and prostaglandin E_2 on the adenylate cyclase activity of plasma membrane fractions of corpus luteum was determined. The final concentration of luteinizing hormone or prostaglandin E_2 in these assays was 10 μg per ml. During these studies the effect of methanol on adenylate cyclase activity of plasma membranes was also evaluated. Methanol, a diluent for prostaglandin E_2 had a final concentration of 0.2% in the adenylate cyclase assay tubes. The effect of GTP, $1 \cdot 10^{-4}$ M, on basal and stimulated adenylate cyclase activities of plasma membrane fractions was also determined.

The activity of 5′-nucleotidase (EC 3.1.3.5), a plasma membrane marker enzyme [13—16] was determined at pH 7.2 by the method of Emmelot and Bos [17]. The inorganic phosphate released in this assay was measured by the method of Fiske and SubbaRow [18]. Succinate dehydrogenase (EC 1.3.99.1), a mitochondrial marker was assayed according to Green et al. [19]. The homogenate and fractions assayed for succinate dehydrogenase activity were preincubated with a solution (5%) of digitonin (9 : 1, v/v) at 4°C for 15 min. Acid phosphatase (EC 3.1.3.2), a lysosomal marker was assayed at pH 4.8 using *p*-nitrophenyl phosphate as the substrate [20]. The activity of microsomal

marker, NADPH-cytochrome *c* reductase (EC 1.6.2.3) was determined according to Phillips and Langdon [21]. Glucose-6-*P* dehydrogenase (EC 1.1.1.49) a cytosol marker was determined according to Durham and Adams [22]. All enzymic assays were conducted under linear conditions with respect to time and concentration of enzymes. The nuclear marker, DNA was determined by the diphenylamine method [23]. Protein concentration was determined by the method of Lowry et al. [24].

Electron microscopy

An aliquot from pellet 2-I_1 (P-2-I_1) was fixed in 5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2) for 10 h followed by a post fixation in 2% OsO_4 (in 0.1 M phosphate buffer, pH 7.2) for 2 h. The pellet was then dehydrated, embedded (in Spurr's resin), and thin sectioned. The sections were stained with uranyl acetate followed by lead citrate. The specimens were examined on an AEI 801 microscope by Dr Robert L. Gulley of the Department of Biological Structure of the University of Miami School of Medicine.

Results

Homogenization and fractionation under isotonic conditions

The percentage distribution of NaF-stimulated adenylate cyclase, the marker enzymes and DNA in different fractions prepared under isotonic conditions of homogenization and fractionation is shown in Table I. Adenylate cyclase was found to be distributed in all the five pellets, but none was present

TABLE I

THE PERCENTAGE DISTRIBUTION OF NaF-STIMULATED ADENYLATE CYCLASE, MARKER ENZYMES AND DNA IN BOVINE CORPUS LUTEUM FRACTIONS PREPARED UNDER ISOTONIC CONDITIONS OF HOMOGENIZATION AND FRACTIONATION

Approx. 5 g of minced corpus luteum tissue were homogenized (1 : 3, w/v) in an isotonic homogenizing medium (0.25 M sucrose, $3 \cdot 10^{-3}$ M $MgCl_2$ and 0.05 M Tris, pH 7.4). The homogenate was separated into fractions as shown in Fig. 1. The fractions were: 250 × *g* for 7.5 min pellet (P-1), 1560 × *g* for 10 min pellet (P-2), 5000 × *g* for 10 min pellet (P-3), 8000 × *g* for 25 min pellet (P-4), 105000 × *g* for 60 min pellet (P-5), and 105000 × *g* for 60 min supernatant (S). The fractions were assayed for adenylate cyclase, marker enzymes and DNA. The final concentration in the reaction mixture (0.61 ml) for adenylate cyclase was Tris (pH 7.4), 39.3 mM; theophylline, 40.0 mM; glycylglycine (pH 7.4), 13.1 mM; $MgSO_4$, 6.56 mM; ATP containing 10 μCi [α-^{32}P] ATP, 1.97 mM; bovine serum albumin, 0.2 mg/ml; NaCl, 2.52 mM, and NaF, 0.01 M. The reaction was carried at 30°C for 10 min in a Dubnoff metabolic shaking incubator. Each value represents mean of three experiments.

Enzyme or marker	Percent of initial homogenate total activity or amount in fractions						Total recovery (%)
	P-1	P-2	P-3	P-4	P-5	S	
Adenylate cyclase	6.0	8.6	12.6	9.7	21.7	0.0	58.6
5'-Nucleotidase	3.4	13.4	16.6	14.8	23.6	0.0	71.8
Acid phosphatase	4.7	5.3	8.7	10.5	19.6	24.7	73.5
Succinate dehydrogenase	3.3	4.4	50.6	30.5	8.4	0.0	97.2
NADPH-cytochrome *c* reductase	1.0	5.2	21.3	17.2	37.3	0.0	82.0
Glucose-6-*P* dehydrogenase	0.0	0.0	0.0	0.0	0.5	76.7	77.2
DNA	43.4	41.1	9.2	4.7	4.0	0.0	102.4

in the supernatant. Pellet 5 which is usually considered to be a microsomal fraction contained most of the recovered adenylate cyclase activity (21.7%). This pellet, however, also contained the highest amount of the plasma membrane marker, 5′-nucleotidase (23.6%) as well as the microsomal marker, NADPH-cytochrome *c* reductase (37.3%) indicating that it was composed of fragments of both plasma membranes and endoplasmic reticulum. Thus on the basis of these experiments alone, adenylate cyclase could have been localized on either of these two subcellular components. The nuclei, the mitochondria, the lysosomes or the cytosol did not appear to be associated with adenylate cyclase.

Homogenization and fractionation under hypotonic conditions

The percentage distribution of NaF-stimulated adenylate cyclase, the marker enzymes and DNA in different fractions prepared under hypotonic conditions of homogenization and fractionation is shown in Table II. Most of the adenylate cyclase activity was present in pellet 2 and 3. No adenylate cyclase was present in the supernatant. The subfractionation of pellet 2 by the discontinuous sucrose gradient centrifugation yielded 8.4, 2.7 and 2.4% of total adenylate cyclase activity at I_1 (d = 1.13), I_2 (d = 1.16) and I_3 (d = 1.20) interphases, respectively. This indicated that only 40.7% of the total adenylate cyclase of pellet 2 was recovered*. Most of this loss seemed to be due to procedural losses rather than enzyme inactivation since only 50.4% of the total protein content of pellet 2 was also recovered** after subfractionation. Subfractionation of pellet 3 also yielded the highest amount of adenylate cyclase activity at interphase I_1 .

Again the percentage distribution pattern of 5′-nucleotidase appeared to follow that of adenylate cyclase. The fraction, P-2-I_1 contained most of the 5′-nucleotidase activity (13.9%) as well as most of the adenylate cyclase activity (8.4%). The distribution of the activities of the other markers did not correspond to that of the adenylate cyclase activity.

The specific activity of NaF-stimulated adenylate cyclase of the different fractions prepared under hypotonic conditions of homogenization and fractionation is shown in Table III. A small degree of purification of adenylate cyclase was achieved under these conditions. Fraction P-2-I_1 and fraction P-3-I_1 had 4.2 and 6.3 times higher respective specific activities compared to that of the homogenate. The specific activity of 5′-nucleotidase in these fractions followed a pattern similar to that of the adenylate cyclase indicating copurification.

Electron microscopy

The electron microscopic examination of the fraction, P-2-I_1 , showed the presence of a homogeneous population of small vesicles ranging from 0.5 to 1 nm (Fig. 3). The fraction exhibited a typical unit membrane structure. Mitochondria, ribosomes of other cytoplasmic contaminants were not found.

---

* Percentage recovery of adenylate cyclase after discontinuous sucrose gradient centrifugation = (sum of the activity of three interphases/activity of pellet 2) × 100 = 13.3/32.7 × 100 = 40.7.

** Percentage recovery of protein after discontinuous sucrose gradient centrifugation was calculated in the same manner as that of the adenylate cyclase mentioned in the preceding footnote.

TABLE II

THE PERCENTAGE DISTRIBUTION OF NaF-STIMULATED ADENYLATE CYCLASE, MARKER ENZYMES, AND DNA IN BOVINE CORPUS LUTEUM FRACTIONS PREPARED UNDER HYPOTONIC CONDITIONS OF HOMOGENIZATION AND FRACTIONATION

Approx. 5 g of minced corpus luteum tissue were homogenized (1 : 4, w/v) in a hypotonic homogenizing medium (1 mM $NaHCO_3$, 5 mM dithiothreitol, pH 7.5). The homogenate was separated into fractions as shown in Fig. 2. The fractions were: 6300 × *g* for 10 min pellet (P-1), 10300 × *g* for 30 min pellet (P-2), 28700 × *g* for 30 min pellet (P-3), and 28700 × *g* for 30 min supernatant (S). The subfractionation of pellet P-2 on discontinuous sucrose gradient separated it into three fractions (P-2-I_1, P-2-I_2, and P-2-I_3). The fractions were assayed for adenylate cyclase, marker enzymes and DNA. The number of separate experiments shown in parentheses was from 2 to 5. Mean values are given.

Enzyme or marker	Percent of initial homogenate total activity or amount in fractions				Total recovery (%)	Percent of initial homogenate total activity or amount in subfractions from P-2		
	P-1	P-2	P-3	S		I_1	I_2	I_3
Adenylate cyclase (5)	22.7	32.7	38.2	0.0	93.6	8.4	2.7	2.2
5′-Nucleotidase (5)	16.1	36.5	31.7	0.0	84.3	13.9	6.0	3.2
Acid phosphatase (5)	27.9	29.3	28.0	29.9	115.1	4.6	2.9	0.9
Succinate dehydrogenase (3)	51.4	24.5	10.1	0.0	86.0	0.0	13.5	3.0
NADPH-cytochrome *c* reductase (3)	15.6	18.7	13.1	51.8	99.2	5.1	3.7	1.9
DNA (2)	67.4	6.6	5.4	0.6	80.0	2.1	1.4	1.5

Correlation between different markers and adenylate cyclase

A significant positive correlation (coefficient of correlation, $r = 0.95$) was found between 5′-nucleotidase and adenylate cyclase under both isotonic and hypotonic conditions of homogenization and fractionation (Table IV). No

TABLE III

THE SPECIFIC ACTIVITY OF NaF-STIMULATED ADENYLATE CYCLASE IN BOVINE CORPUS LUTEUM FRACTIONS PREPARED UNDER HYPOTONIC CONDITIONS OF HOMOGENIZATION AND FRACTIONATION

The preparation of fractions under hypotonic conditions of homogenization and fractionation is shown in Fig. 2. Each value represents specific activity (mean ± S.E.).

Fraction	Number of experiments	Specific activity of adenylate cyclase (pmol cyclic AMP · min^{-1} · mg^{-1} protein)
Homogenate	5	13.4 ± 4.7
6300 × *g* for 10 min pellet (P-1)	5	16.5 ± 4.2
10300 × *g* for 30 min pellet (P-2)	5	35.4 ± 9.0
28700 × *g* for 30 min pellet (P-3)	5	64.1 ± 12.5
Subfractions from P-2:		
P-2-I_1	5	56.8 ± 15.0
P-2-I_2	5	15.6 ± 6.3
P-2-I_3	5	31.3 ± 7.3
Subfractions from P-3:		
P-3-I_1	2	83.9 ± 6.9
P-3-I_2	2	41.6 ± 1.1
P-3-I_3	2	32.8 ± 19.6

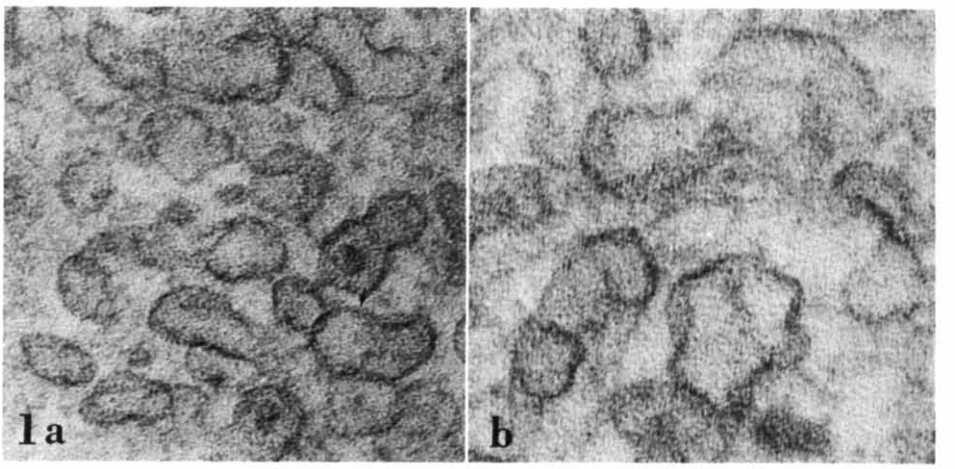

Fig. 3. A representative field of plasma membrane fraction (P-2-I_1) at a magnification of 54 000-fold (A) and 75 000-fold (B). An aliquot from fraction P-2-I_1 was fixed in glutaraldehyde. The fixation was followed by a post-fixation in OsO_4. The pellet was dehydrated embedded and sectioned. The sections were stained with uranyl acetate and lead citrate. The unit membrane structure is visible at the arrow. Microsomes or other cytoplasmic contaminants were absent. The membranes showed a homogeneous population of vesicles ranging from 0.5 to 1.0 nm.

other marker was significantly correlated with adenylate cyclase under both these conditions. A positive correlation (r = 0.94) between NADPH-cytochrome *c* reductase and adenylate cyclase was found under isotonic condition of fractionation, however, no relationship (r = —0.09) between these two enzymes was revealed by the data obtained on fractions prepared under hypotonic conditions of fractionation. A possible correlation (r = 0.62) between acid phosphatase and adenylate cyclase was suggested by the data obtained on fractions prepared under hypotonic conditions but no relationship (r = —0.02)

TABLE IV

CORRELATION BETWEEN DIFFERENT MARKERS AND ADENYLATE CYCLASE

The coefficients of correlation (r) between different markers and adenylate cyclase were calculated as outlined by Steel and Torrie [25]. The mean values of the percent distribution of the different markers and adenylate cyclase in the fractions obtained by both isotonic and hypotonic procedures were used to calculate these coefficients.

Marker versus adenylate cyclase	r Values under different conditions of homogenization and fractionation		
	Isotonic (n = 6)	Hypotonic (n = 7)	Pooled data (n = 13)
5′-Nucleotidase	0.95*	0.95*	0.95**
NADPH-cytochrome *c* reductase	0.94*	−0.09	0.16
Succinate dehydrogenase	0.30	0.47	0.37
Acid phosphatase	−0.02	0.62	0.53
DNA	−0.19	0.29	0.14
Glucose-6-*P* dehydrogenase	−0.65	—	—

* r differs from zero $P < 0.05$.
** r differs from zero $P < 0.01$.

TABLE V

EFFECT OF LUTEINIZING HORMONE, NaF, PROSTAGLANDIN E_2 AND METHANOL ON ADENYLATE CYCLASE ACTIVITY IN PLASMA MEMBRANES OF BOVINE CORPUS LUTEUM

The preparation of plasma membrane fractions is described in Materials and Methods section. Each value represents mean ± S.E. The number in parentheses indicates the number of experiments conducted. The comparison between a treatment and the corresponding basal (control) was made by Student's *t*-test [26]. n.d., not determined.

Treatment	Adenylate cyclase activity (pmol cyclic AMP · min^{-1} · mg^{-1} protein) in plasma membrane fractions		
	P-2-I_1	P-3-I_1	P-2+3-I_1
Basal	14.2 ± 3.9 (4)	11.2 ± 1.2 (4)	14.4 ± 2.8 (4)
Luteinizing hormone, 10 μg/ml	37.5 ± 7.5* (4)	37.0 ± 9.8* (4)	41.4 ± 5.3* (4)
NaF, 0.01 M	66.1 ± 8.1** (9)	78.6 ± 14.6** (6)	111.8 ± 11.7** (4)
Prostaglandin E_2, 10 μg/ml + methanol 0.2%	n.d.	n.d.	56.3 ± 7.4** (4)
Methanol, 0.2%	n.d.	n.d.	28.3 ± 5.4*** (4)

* Differs from the corresponding basal control, $P < 0.05$.
** Differs from the corresponding basal control, $P < 0.01$.
*** Apparently differs from the corresponding basal control, $P < 0.10$.

between these two enzymes was indicated by the data obtained on fractions prepared under isotonic conditions (Table IV).

Effect of luteinizing hormone, NaF and prostaglandin E_2

It was of interest, to determine if the adenylate cyclase activity of plasma membrane fractions would respond to agents such as NaF, luteinizing hormone or prostaglandin E_2 which had been shown to stimulate adenylate cyclase in homogenates of corpora lutea [2,3]. The adenylate cyclase activity (Table V) in all these fractions was stimulated by luteinizing hormone (2—3-fold) and NaF (4—8-fold). Prostaglandin E_2 dissolved in methanol significantly stimulated (3—4-fold) the adenylate cyclase activity of plasma membrane fraction P-2+3-I_1. Part of this effect seemed to be due to the solvent since methanol (0.2%) also appeared to stimulate adenylate cyclase activity of plasma membranes about 2-fold. The addition of GTP ($1 \cdot 10^{-4}$ M) had no effect on basal, or stimulated adenylate cyclase activities of plasma membrane fractions P-2-I_1, P-3-I_1 or P-2+3-I_1.

Discussion

When the corpus luteum fractions were prepared under isotonic conditions there was a high correlation of the adenylate cyclase activity with the marker enzymes for both plasma membranes (5'-nucleotidase) and the endoplasmic reticulum (NADPH-cytochrome *c* reductase), indicating that the adenylate cyclase could be associated with either or both of these subcellular organelles. Under these isotonic conditions the highest amount of adenylate cyclase also appeared in the fraction where fragments of endoplasmic reticulum are usually expected to accumulate, but this fraction was apparently contaminated to a large extent by fragments of plasma membranes as judged by the

amount of 5′-nucleotidase detected. A similar phenomenon was observed by House et al. [27] during the isolation of plasma membranes from rat liver under isotonic conditions (buffered 0.25 M sucrose). They reported the isolation of a microsomal fraction which contained 30—35% of the total crude homogenate 5′-nucleotidase activity. El-Aasser and Reid [28] reported that even with the mildest homogenization only one half of the 5′-nucleotidase activity was associated with crude nuclear fraction, the remainder was associated with the microsomal fraction.

In the studies where the fractions were prepared under hypotonic conditions the distribution of adenylate cyclase only correlated well with the plasma membrane marker enzyme (5′-nucleotidase). There was some correlation between the adenylate cyclase and the lysosomal marker enzyme (acid phosphatase), but the possible localization of adenylate cyclase with lysosomes was ruled out by the data obtained from isotonic fractionation mentioned earlier. Similarly, the possibility that adenylate cyclase is associated with the endoplasmic reticulum, as suggested by the isotonic studies, was ruled out by the data obtained with hypotonic fractionation. The only possibility that we are left with then is that adenylate cyclase of this tissue is associated with plasma membranes. This interpretation is arrived at not only because adenylate cyclase correlated extremely well with a classical enzyme marker for plasma membranes [13–16], but also because the markers for the other organelles did not correlate with adenylate cyclase. A further piece of evidence was that an electron micrograph of one of the fractions with a high adenylate cyclase specific activity revealed only membrane fragments with no indication of the presence of ribosomes or other cytoplasmic contaminants. It is concluded, therefore, that the adenylate cyclase of bovine corpora lutea is primarily if not exclusively localized on the plasma membrane.

Rao and Saxena [29] have also concluded that adenylate cyclase is predominantly localized on the plasma membranes obtained from luteinized rat ovaries. This conclusion was, however, based on a limited amount of fractionation data in which no biochemical characterization of the fractions by standard markers was reported. Menon and Kiburz [6] have recently reported the existence of adenylate cyclase in a "plasma membrane" fraction obtained from bovine corpora lutea, but the purpose of this study was not to determine the subcellular localization of the adenylate cyclase. No attempt was made to ascertain if their "plasma membrane" fraction was the primary localization of this enzyme or to what extent this fraction was contaminated by other organelles.

The level of stimulation of adenylate cyclase activity of plasma membranes of corpora lutea by luteinizing hormone (Table V) is just about the same as that observed previously in whole homogenates of bovine corpora lutea [2,3]. Recently, Menon and Kiburz [6] also reported a 3-fold stimulation of adenylate cyclase activity of a "plasma membranes" fraction of bovine corpora lutea by human chorionic gonadotropin. The stimulation of adenylate cyclase by prostaglandin E_2 indicates the presence of a prostaglandin E_2-sensitive adenylate cyclase in the plasma membranes of corpora lutea. The stimulatory effect of this prostaglandin on cyclic AMP accumulation has been reported in a variety of ovarian tissue, but the biological significance of this effect is obscure

[3,30—37]. NaF produced a greater stimulation of adenylate cyclase activity in the plasma membrane fraction of this tissue than either luteinizing hormone or prostaglandin E_2. The fluoride stimulation of adenylate cyclase activity has been consistently observed in the study of luteinizing hormone and other hormones [2,36,38]. The stimulation of adenylate cyclase activity by methanol appears to be a non-specific effect because a similar effect of ethanol on adenylate cyclase activity in plasma membranes of rat liver and kidney and bovine thyroid has been reported [39]. It was suggested that this effect may be due to a perturbation of the plasma membrane structure [39].

In conclusion, it is reported that adenylate cyclase of bovine corpus luteum is primarily localized in the plasma membranes. The adenylate cyclase activity of isolated plasma membranes is stimulated by luteinizing hormone, prostaglandin E_2, NaF and methanol.

Acknowledgements

We are grateful to Dr Robert L. Gulley for electron microscopic studies, to the National Institute of Arthritis and Metabolic Diseases for generous supply of gonadotropins, to Dr John Pike, The Upjohn Co. for gift of prostaglandins, to Mr Jorge Cidre and Mrs Adalgisa Rojo for excellent technical assistance, and to Mrs Nieves Cerver and Mrs Narvinder Sidhu for help in the preparation of the manuscript. This work was supported in part by National Institutes of Health Grant HD 03142 and Ford Foundation Grant 0338.

References

1 Savard, K., Marsh, J.M. and Rice, B.F. (1965) Recent Prog. Hormone Res. 21, 285—364
2 Marsh, J.M. (1970) J. Biol. Chem. 245, 1596—1603
3 Marsh, J.M. (1970) FEBS Lett. 7, 283—286
4 Marsh, J.M., Butcher, R.W., Savard, K. and Sutherland, E.W. (1966) J. Biol. Chem. 241, 5436—5440
5 Marsh, J.M. and Savard, K. (1966) Steroids 8, 133—148
6 Menon, K.M.J. and Kiburz, J. (1974) Biochem. Biophys. Res. Commun. 56, 363—371
7 Danzo, B.J., Midgley, Jr, A.R. and Kleinsmith, L.J. (1972) Proc. Exp. Biol. Med. 139, 88—92
8 Lee, C.Y. and Ryan, R.J. (1973) Adv. Exp. Med. Biol. 36, 419—430
9 Kristoffersen, J. (1960) Acta Endocrinol. 33, 417—427
10 Neville, Jr, D.M. (1960) J. Biophys. Biochem. Cytol. 8, 413—422
11 Krishna, G., Weiss, B. and Brodie, B.B. (1968) J. Pharmacol. Exp. Ther. 163, 379—385
12 Bray, G.A. (1960) Anal. Biochem. 1, 279—285
13 Essner, E., Novikoff, A.B. and Masek, B. (1958) J. Biophys. Biochem. Cytol. 4, 711—715
14 Touster, O., Aronson, Jr, N.N., Dulaney, J.T. and Hendrickson, H. (1970) J. Cell Biol. 47, 604—618
15 Hinton, R.H. (1972) Subcellular Components; Preparation and Fractionation (Birnie, G.D., ed.), pp. 119—156, University Park Press, Baltimore
16 Gospodarowicz, D. (1973) J. Biol. Chem. 248, 5050—5056
17 Emmelot, P. and Bos, C.J. (1966) Biochim. Biophys. Acta 120, 369—382
18 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
19 Green, D.E., Mii, S. and Kohout, P.M. (1955) J. Biol. Chem. 217, 551—567
20 Linhardt, K. and Walter, K. (1963) Methods of Enzymatic Analysis (Bergmeyer, H.V., ed.), pp. 779—787, Academic Press, New York
21 Phillips, A.H. and Langdon, R.G. (1962) J. Biol. Chem. 237, 2652—2660
22 Durham, N.N. and Adams, L.S. (1966) Biochim. Biophys. Acta 121, 90—101
23 Burton, K. (1956) Biochem. J. 62, 315—323
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
25 Steel, R.G.D. and Torrie, J.H. (1960) Principles and Procedures of Statistics, pp. 183—193, McGraw-Hill Book Co., Inc., New York

26 Steel, R.G.D. and Torrie, J.H. (1960) Principles and Procedures of Statistics, pp. 67—87, McGraw-Hill Book Co., Inc., New York
27 House, P.D.R., Poulis, P. and Weidemann, M.J. (1972) Eur. J. Biochem. 24, 429—437
28 El-Aasser, A.A. and Reid, E. (1969) Histochem. J. 1, 417—437
29 Rao, C.V. and Saxena, B.B. (1973) Biochim. Biophys. Acta 313, 372—389
30 Kuehl, Jr, F.A., Humes, J.L., Tarnoff, J., Cirillo, V.J. and Ham, E.A. (1970) Science 169, 883—886
31 Marsh, J.M. (1971) Ann. N.Y. Acad. Sci. 180, 416—425
32 Kolena, J. and Channing, C.P. (1972) Endocrinology 90, 1543—1550
33 Channing, C.P. (1972) Prostaglandins 2, 351—367
34 Lamprecht, S.A., Zor, U., Tsafriri, A. and Lindner, H.R. (1973) J. Endocrinol. 57, 217—233
35 Jonsson, Jr, H.T., Shelton, V.L. and Baggett, B. (1973) in Prostaglandins and Cyclic AMP. Biological Actions and Clinical Applications (Kahn, R.H. and Lands, W.E.M., eds), pp. 183—186, Academic Press, New York
36 Marsh, J.M. and LeMaire, W.J. (1974) J. Clin. Endocrinol. Metab. 38, 99—106
37 Kuehl, Jr, F.A. (1974) Prostaglandins 5, 325—340
38 Perkins, J.P. (1973) Advances in Cyclic Nucleotide Research (Greengard, P. and Robinson, G.A., eds), Vol. 3, pp. 1—64, Raven Press Publishers, New York
39 Mashiter, K., Mashiter, G.D. and Field, J.B. (1974) Endocrinology 94, 370—376
40 Sidhu, K.S., Camp, C.E. and Marsh, J.M. (1974) Fed. Proc. Fed. Am. Soc. Exp. Biol. 33, 204 Abstr.