Expression of rat mRNA coding for hormone-stimulated adenylate cyclase in *Xenopus* oocytes

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**ABSTRACT**

β-Adrenergic agonist-stimulated hyperpolarization, whole-cell cAMP accumulation, and activity of isoproterenol-stimulated membrane-bound adenylate cyclase (EC 4.6.1.1) in *Xenopus laevis* ovarian oocytes are entirely dependent on the presence of nascent follicle cells. A method was developed to remove rapidly and completely all extra-oocyte cell types to yield defolliculated oocytes that exhibited normal viability and resting membrane potentials yet lacked β-adrenergic receptor (βAR)-stimulated responses. Purified follicle membranes contained βAR-stimulated adenylate cyclase activity, whereas oocyte cell membranes did not. Purified oocyte membrane preparations from *X. laevis* oocytes previously microinjected with C6-2B rat astrocytoma mRNA, and subsequently defolliculated, exhibited novel βAR and forskolin-stimulated adenylate cyclase activity. These experiments demonstrate that oocytes expressed rat C6-2B mRNA coding for the β-adrenergic receptor and the components necessary for forskolin-stimulated adenylate cyclase activity. — SMITH, A. A.; BROOKER, T.; BROOKER, G. Expression of rat mRNA coding for hormone-stimulated adenylate cyclase in *Xenopus* oocytes. FASEB J. 1: 380–387; 1987.

**Key Words:** *Xenopus laevis* oocyte · follicle cells · adenylate cyclase · hyperpolarization · β-adrenergic receptor

Our laboratory has proposed that a rapidly turning over regulatory protein or proteins are involved in the development of catecholamine-induced refractoriness (1). In our quest to express and ultimately identify these proteins involved in refractoriness, we have developed the *Xenopus laevis* oocyte mRNA translation system for the functional expression of rat C6-2B astrocytoma cell (2) mRNA coding for the β-adrenergic receptor (βAR) and the components of adenylate cyclase (EC 4.6.1.1) responsible for forskolin activation of the enzyme.

Individual ovarian oocytes, within the ovarian lobes in situ, are encapsulated by follicles that consist of, in order of proximity to the oolemma/oocyte vitelline membrane complex, follicle cells, the theca, a col-lagenous intercellular matrix containing fibroblasts and small blood vessels, and the outermost simple squamous peritoneal epithelium of the ovary (3). The follicle acts to maintain the stage 6 oocyte arrested in prophase of the first meiotic division (4), as well as to mediate steroid biosynthesis in response to hormonal stimulation (5, 6), vitellogenin uptake (7), and amino acid uptake (8) during the earlier stages of oocyte development. Active gap junctions have been identified between *X. laevis* follicle cells and oocytes isolated from toads preinjected with human chorionic gonadotropin (9). The presence of such low-resistance junctions allows ionic coupling and synchronous electrical activity in many tissues, including the heart, as well as in co-cultures of mouse granulosa cells with myocardial cells (10). Inasmuch as the *X. laevis* ovarian oocyte is both electrically (11) and biochemically responsive to the β-adrenergic agonist isoproterenol and to the adenylate cyclase activator forskolin, it was of critical importance to determine in which cellular membranes these endogenous responses originated. Thus, to elucidate the role of the follicle in ovarian oocyte hormone responsiveness, we developed a method to remove completely from the ovarian oocyte the encapsulating follicle cells and surrounding thecal cell layers. This defolliculation method involves sequential treatment by a low concentration of digitonin, collagenase, and manual removal of any remaining loosely attached cell layers.

Our initial electrophysiological data supported other studies (11-13) that demonstrated an endogenous hyperpolarization response in the ovarian oocyte. Ovarian oocytes were hyperpolarized by isoproterenol, forskolin, and 8-bromo-cAMP. The isoproterenol-induced hyperpolarization is β adrenergic in character because it is blocked completely by the βAR antagonist (-)-propranolol (12). The adenylate cyclase activator forskolin has also been shown to induce membrane hyperpolarization by a similar mechanism and to potentiate the

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isoproterenol-induced hyperpolarizing K⁺ efflux current (13). In our experiments, none of these agents provoked a hyperpolarization response in oocytes defolliculated according to our new method.

Because the isoproterenol and forskolin responses depend on the presence of the follicle, adenylate cyclase activity in membranes from mRNA-injected defolliculated oocytes provides a means of detecting functional expression of mRNA coding for the βAR and other adenylate cyclase components. We report here the functional expression in oocytes of C6-2B cell mRNA coding for isoproterenol and forskolin-stimulated adenylate cyclase.

MATERIALS AND METHODS

Oocyte isolation

Adult X. laevis females were obtained from Carolina Biologicals (Burlington, NC), fed twice a week with calves liver, and maintained in tanks containing 2 liters fresh aerated tap water per animal. Ovarian oocytes were surgically removed from non-gonadotropin-stimulated dormant females that had been anesthetized for 20 min in ice water (11). One to ten lobes of the ovary were removed by laparotomy. The surgical incision was sutured with 6.0 Coated Vicryl Ophthalmic absorbable suture (Ethicon, Sommerville, NJ) and the toad was allowed to recover for future experiments. The ovarian lobes were immediately rinsed in 21°C Ringer's solution [0.82 mM MgSO₄, 0.74 mM CaCl₂, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 88 mM NaCl, 2.4 mM NaHCO₃, and 10 mM Tris-HCl (pH 7.6)], or Modified Barth's Solution [0.82 mM MgSO₄, 0.41 mM CaCl₂, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 88 mM NaCl, 2.4 mM NaHCO₃, and 10 mM HEPES] [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6)], containing 2.5 mM sodium pyruvate (Gibco, Grand Island, NY) and filtered using a sterile 0.22-µm Nalgene filter. Stage 5 oocytes, according to Dumont (3), were manually isolated from ovarian lobes by using jeweler's forceps under a dissecting microscope. Oocytes were maintained at 21°C in a temperature-regulated incubator with fresh medium added at least every 12 h. Optimal oocyte longevity was observed in pyruvate-containing Modified Barth's Solution.

Cell culture

Monolayer cultures of C6-2B rat astrocytoma cells (passage 10–30) were grown at 37°C in 150-mm tissue culture plates in Ham's F-10 nutrient medium (Gibco, Grand Island, NY) buffered with 14 mM NaHCO₃ (pH 7.4), supplemented with 10% donor calf serum in a humidified atmosphere of 5% CO₂/95% air. The cells were re-fed on day 4 and harvested on day 6 or 7 while subconfluent and in the midlogarithmic phase of growth.

Poly(A)' RNA isolation

The guanidine thiocyanate method of Chirgwin et al. (14) was used to isolate RNA from cells. In addition, the RNA was cycled twice over an oligo(dT) column to isolate poly(A)' mRNA (15). To inactivate ribonuclease, all glassware was rinsed with 5 M NaOH and distilled water, siliconized using PROSIL (SCM Chemicals, Gainesville, FL) organosilane according to the manufacturer's directions (to reduce RNA to glass adherence), rinsed with 0.1% diethyl pyrocarbonate, autoclaved, and baked overnight at 250°C. Solutions were prepared with double distilled water that had been pretreated with 0.1% diethyl pyrocarbonate, heated overnight at 70°C, and autoclaved twice to ensure the removal of the volatile diethyl pyrocarbonate. C6-2B cells were rinsed, scraped from the plastic plates, and pelleted at 5000 × g in cold phosphate-buffered saline (Dulbecco's). The cellular pellets were lysed with 10 strokes of a Dounce B pestle homogenizer at 21°C after the addition of 4 mM guanidinium thiocyanate (GTC), 25 mM sodium citrate (pH 7.0), containing freshly added 100 mM β-mercaptoethanol. The cellular lysates were layered over 5.7 M CsCl containing 0.1 M EDTA (pH 7.0) in polyalloymer ultracentrifuge tubes and centrifuged for 18 h at 33,000 rpm in a SW 40 Ti rotor (Beckman, Palo Alto, CA) at 22°C. The supernatant was aspirated leaving a 1-cm CsCl layer. The walls of the tubes were rinsed three times each with 0.5 ml of GTC, the remaining CsCl was decanted, and the bottom 1 cm of the tube was cut. The RNA pellets were then rinsed with −20°C 80% ethanol containing 0.25 M sodium acetate (filtered 0.45 µM), lyophilized, and resuspended in 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA (TE solution). The RNA was then precipitated at −20°C for 24 h after the addition of sodium acetate to a final concentration of 0.3 M followed by addition of 2.2 volumes 0°C 100% ethanol (filtered 0.45 µM). Lyophilized ethanol precipitates were resuspended in 65°C TE for 5 min, cooled to 0°C, and applied to 1 ml containing 50 mg of prequillibrated oligo(dT) [Collaborative Research Oligo(dT)-Cellulose Type 3, Lexington, MA] in a 1.5-ml Eppendorf microcentrifuge tube. The RNA was mixed by inversion and centrifuged for 1 min using a Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY). The first supernatant fraction was re-heated and reapplied. Poly(A)' RNA was first eluted using 0.01 M Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. After a 0.1 M NaCl-containing wash, the poly(A)' RNA was eluted in 45°C buffer containing no salt. Then 4 M sodium acetate was added to the eluted poly(A)' RNA fractions to yield a final concentration of 0.2 M sodium acetate. The mRNA was precipitated by the addition of 2.5 volumes of 0°C 100% ethanol (filtered 0.45 µM) and stored at −70°C.

Oocyte microinjection

Our microinjection apparatus consisted of a dissecting microscope in conjunction with a computerized system for control of the air-actuated injection pipet that was positioned by a micromanipulator (manuscript in preparation). A television camera with a microscopic lens was used to monitor the position of the meniscus on the injection pipet, and thus the volume delivered by microinjection could be readily calculated. In concept,

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our microinjection system is similar to that reported previously (16). The pipet tip diameter varied from 3 to 30 μm depending on the experiment. Approximately 50 nl of Ringer's solution or 1 mg/ml C6-2B mRNA was injected into each stage 5 oocyte (17).

Oocyte defolliculation

Defolliculation was accomplished by pretreatment with 0.01% digitonin at 21°C for 5 min with occasional inversion in a 50-ml polypropylene tube containing 50 oocytes per 50 ml, followed by three 50-ml rinses, and 30 min incubation in Sigma type II collagenase (Sigma Chemical Co., St. Louis, MO) (5 mg/ml) at 21°C that had been dialyzed against Ringer's or Modified Barth's Solution. Microscopic examination of these oocytes showed a halolike lifting off of the follicle. Using newly refinished fine-tipped jeweler's forceps, the follicles were removed in one piece by gently peeling away the slightly separated tissue.

Oocyte electrophysiology

Oocyte membrane potentials were measured with a World Precision Instruments (WPI; New Haven, CT) M-707 microprobe system using beveled 3 M KCl-filled glass microelectrodes made from (0.58 mm i.d., 1.0 mm o.d.) 1B100F WPI Kwik-Fil capillary tubing. The microelectrodes were pulled on a David Kopf Instruments Model 700C Vertical Pipette Puller (Tujunga, CA) and had more than 10 MΩ resistance. The superfusion bath consisted of a 800-μl polypropylene chamber containing a polypropylene screen to support the oocyte. The superfusion solution was pumped by a peristaltic pump at 1.2 ml/min from the base of the chamber and bathed the submerged oocyte. For transient drug application, 200 μl of drug in Ringer's or Barth's solution was added directly to the chamber during constant superfusion. Alternatively, for prolonged superfusion, the drug was continuously pumped into the chamber.

Membrane purification

Ovarian oocytes, defolliculated oocytes, and isolated follicles were homogenized on ice for 6 s at 27,000 rpm with a Polytron (Brinkmann Instruments, Westbury, NY; PTA-7) in one volume, or, in the case of the isolated follicles, 200 follicles per 200 μl of 0.88 M sucrose, 50 mM Tris-HCl (pH 8.0), 0.2 mM dithiothreitol (DTT), and 1 mM EGTA. The cellular lysates were centrifuged at 8000 × g for 10 min at 4°C. The upper bright yellow yolk lipid aspirate was discarded and the supernatant solution from the follicle and the predominant middle layer of the oocyte preparations were transferred to clean centrifuge tubes and centrifuged at 20,000 × g for 20 min at 4°C. The resulting supernatant solutions were then centrifuged at 105,000 × g for 2 h. The fluffy pellets were resuspended in 50 mM Tris-HCl (pH 7.4) containing 0.2 mM DTT and centrifuged again twice to ensure the removal of EGTA (18).

Adenylate cyclase assay

Adenylate cyclase activity was measured at 30°C for 30 min with 2-50 μg membrane protein in a volume of 10-50 μl containing 50 mM Tris-HCl (pH 7.6), 11 mM MnCl₂, 1 mM EDTA, 10 μM GTP, 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM ATP, and an ATP-regenerating system containing 25 mM creatine phosphate, 25 U/ml creatine phosphokinase, pH 7.6, essentially according to the method of Stengel and Hánoune (19). The reaction was terminated with 10 volumes of 0.1 N HCl. cAMP generated by adenylate cyclase was then assayed as described below.

cAMP determination

Whole oocytes (usually 1 oocyte/0.5 ml) were incubated in Modified Barth's Solution containing 1 mM 3-isobutyl-1-methylxanthine at room temperature in the presence or absence of isoproterenol and/or forskolin. The reaction was stopped at the indicated times by the addition of one-tenth volume 1 N HCl and heating for 3 min at 95°C. The cAMP content of whole-oocyte HCl extracts or of acidified adenylate cyclase assay samples, prepared as described above, was measured with the Gamma-Flo Automated Radioimmunoassay System (Atto Instruments, Potomac, MD) operating at 60 samples/h (20) after acetylation as described by Harper and Brooker (21).

Protein determination

Protein concentrations were determined by the Bradford dye-binding method (22) using a microplate reader.

RESULTS

Isolation of oocytes free of supportive cell layers

In our hands, previously reported methods designed to remove the follicle from ovarian oocytes (4, 11, 23-25) resulted in the incomplete removal of the extra-oocyte cell layers or yielded denuded oocytes that exhibited markedly diminished resting membrane potentials. For this reason, we developed a method of defolliculation, using a sequential combination of digitonin and collagenase that renders the oocyte free of adherent follicle cells. Two standard collagenase methods for follicle cell removal (11, 24) did not always result in the complete dissociation of the closely adherent follicle cells from the oocyte plasma membrane/vitelline membrane complex. The sequential treatment of ovarian oocytes with digitonin and collagenase (Fig. 1, A-D) results in the complete removal of the nonoocyte supportive cell layers. Figure 1B shows the disruption of the extra-oocyte cellular matrix and the permeabilization of the great majority of all cells within the follicle layer after digitonin treatment (26). Light microscopic observation of the follicle layer provided further evidence that these cells were permeabilized by digitonin treatment because the vital exclusion dye trypan blue revealed essentially

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Figure 1. Composite electron micrographs of oocytes, control or after various treatments designed to accomplish defolliculation (× 14,600 final magnification, slightly reduced in reproduction). A) Ovarian oocytes showing the oocyte cytoplasm filled with yolk platelet granules (O), the oocyte plasma membrane, the proteinaceous oocyte vitelline membrane (V), adjacent follicle cell (F), and surrounding thecal layer (T) consisting of a collagenous intercellular matrix, fibroblast cells, and a capillary network. B) Ovarian oocyte after 5-min treatment with 0.01% digitonin. C) Ovarian oocyte after 30-min incubation with 5 mg/ml collagenase type II. D) Completely defolliculated oocyte showing the oocyte plasma membrane surrounded by the oocyte vitelline membrane after a sequential treatment with digitonin followed by collagenase, as described, followed by manual removal of the separated follicle tissue.

no intact cells, and no dye was taken up by the oocyte (data not shown). Collagenase (5 mg/ml) treatment alone for 30 min did not result in complete defolliculation (Fig. 1C). Digitonin pretreatment facilitates the action of collagenase because the time required for the collagenase-induced disruption of the adherent follicle cell layer in digitonin-treated oocytes required only 30 min (Fig. 1D) compared with the 4–18 h required when oocytes were not pretreated with digitonin.

Hormone-stimulated elevation of cAMP in ovarian oocytes is follicle dependent

After a 30-min incubation in the presence of isoproterenol and forskolin, whole ovarian oocyte cAMP levels were increased approximately 15-fold over basal (Table 1). This response was almost totally obliterated after pretreatment of oocytes with digitonin, but only partially decreased after collagenase pretreatment. After the sequential treatment of ovarian oocytes with both digitonin and collagenase, the resulting defolliculated oocytes no longer responded to isoproterenol or forskolin.

Isoproterenol-induced hyperpolarization in ovarian oocytes is follicle dependent

As illustrated in Fig. 2, the follicle-encapsulated oocyte (ovarian oocyte) hyperpolarized on exposure to isoproterenol, forskolin, or a membrane-permeable cAMP analog, 8-bromo-cAMP. We also observed that the time required for 8-bromo-cAMP to initiate hyperpolarization was longer than that of isoproterenol or forskolin and that recovery of the resting membrane potential
TABLE 1. Whole oocyte cAMP response to 10 μM (−)-isoproterenol and/or 100 μM forskolin after no treatment, collagenase 5 mg/ml for 30 min, or 0.01% digitonin for 5 min, or sequential treatment with both digitonin and collagenase

<table>
<thead>
<tr>
<th>Pretreatment Condition</th>
<th>cAMP response (pmol/ml)</th>
<th>Ringer's</th>
<th>Digitonin</th>
<th>Collagenase</th>
<th>Digitin + collagenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringer's</td>
<td>1.60 ± 0.18</td>
<td>1.15 ± 0.17</td>
<td>1.54 ± 0.07</td>
<td>1.49 ± 0.07</td>
<td></td>
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<tr>
<td>Isoproterenol</td>
<td>1.32 ± 0.20</td>
<td>1.54 ± 0.14</td>
<td>1.60 ± 0.08</td>
<td>1.42 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Forskolin</td>
<td>4.03 ± 0.40</td>
<td>2.16 ± 0.30</td>
<td>4.89 ± 0.81</td>
<td>1.72 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Isoproterenol + forskolin</td>
<td>24.84 ± 2.22</td>
<td>2.76 ± 0.47</td>
<td>10.40 ± 1.60</td>
<td>2.12 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>

*Individual oocytes were incubated in 0.5 ml. The cAMP responses are expressed ± SEM for four or five oocytes per condition.

was slower, as had been previously reported (13). However, defolliculated oocytes had membrane resting potentials comparable to ovarian oocytes yet neither 8-bromo-cAMP, isoproterenol, nor forskolin induced a hyperpolarization event. These data suggest that the oocyte membrane does not contain the components necessary for initiation of membrane hyperpolarization by isoproterenol, forskolin, or 8-bromo-cAMP.

β-Adrenergic-coupled cyclase activity is present only in follicle-containing preparations

As shown in Fig. 3, purified membrane preparations from ovarian oocytes, follicle cells, and follicle-free oocytes exhibited basal adenylate cyclase activity. Forskolin (10 μM) plus (−)-isoproterenol (10 μM) stimulated adenylate cyclase activity in the follicle cell-

Figure 2. Electrophysiological intracellular membrane potential recordings of ovarian (left four traces) and defolliculated (right three traces) oocytes on superfusion with 10 μM (−)-isoproterenol, 10 μM forskolin, or 2 mM 8-bromo-cAMP.
containing preparations but did not stimulate follicle-free, defolliculated oocyte membrane preparations. These results substantiate our impression that the oocyte membrane lacks the components necessary for adenylate cyclase activity stimulated by β-adrenergic agonists.

Expression of novel β-adrenergic agonist and forskolin-stimulated adenylate cyclase activity, after microinjection of rat C6-2B mRNA, in purified defolliculated oocyte membrane preparations

Whole defolliculated oocytes that had been microinjected with the C6-2B mRNA exhibited a novel stimulatory response, an elevation in whole oocyte acid extractable cAMP content, to isoproterenol plus forskolin. This response was less than twofold above vehicle microinjected oocytes, 1.83 ± 0.09 vs. 1.11 ± 0.10 pmol cAMP/oocyte in control defolliculated oocytes. To study this novel activity at the level of adenylate cyclase, we prepared purified membranes from hundreds of defolliculated oocytes that had been previously injected with either vehicle or C6-2B mRNA for subsequent assay of in vitro adenylate cyclase activity. As shown in Fig. 1, 1 or 10 μM forskolin stimulated adenylate cyclase activity in membranes prepared from mRNA-injected oocytes about two- to fourfold vs. 1- to 1.4-fold in membranes prepared from vehicle-injected oocytes. Incubation of purified membranes from C6-2B mRNA microinjected defolliculated oocytes, or noninjected ovarian oocytes, in the presence of (-)-isoproterenol alone resulted in a small but statistically significant stimulation of adenylate cyclase activity above basal whereas vehicle-injected defolliculated oocytes did not (data not shown). Notwithstanding, as in whole oocytes (Table 1), the forskolin-potentiated isoproterenol response in membranes from mRNA-injected oocytes was readily measurable (Fig. 4), 495 ± 34% above basal vs. 28 ± 6% above basal in the vehicle-injected defolliculated oocyte membrane preparations.

The potentiated response of 10 μM (-)-isoproterenol by 10 μM forskolin was blocked by 5 μM (-)-propranolol such that the stimulation of adenylate cyclase activity by isoproterenol plus forskolin in the presence of propranolol was equal to that of forskolin alone. Propranolol had no effect on forskolin-stimulated activity.

DISCUSSION

Surgically removed ovarian oocytes are encapsulated by a follicle that we found was responsible for endogenous oocyte βAR-coupled adenylate cyclase activity. Thus the elimination of this endogenous adenylate cyclase activity was pivotal to expression and characterization of exogenous βAR mRNA. It was fortunate that the β-adrenergic response could be localized to the follicle cell layer because we were able to devise a technique to effectively remove this layer from oocytes. Our method of defolliculation allows the removal of extracellular layers such that oocyte viability was unaffected.

Intra-oocyte hyperpolarization to isoproterenol, forskolin, and 8-bromo-cAMP was also observed to be strictly follicle dependent in these studies. The follicle-dependent nature of this response has been postulated based on observations that removal of the follicle usually suppresses the response of oocytes to catecholamines (11, 12), but this has never been systematically studied. Browne et al. (9) reported the presence of gap junctions between the oocyte and follicle cells that, after gonado-

![Figure 3. Basal adenylate cyclase activity and activity induced by 10 μM (-)-isoproterenol plus 10 μM forskolin in purified membrane preparations from ovarian oocytes, isolated follicle cell layers, and defolliculated oocytes.](image)

![Figure 4. Adenylate cyclase activity in C6-2B poly(A)⁺ RNA- or vehicle-microinjected defolliculated oocyte membrane preparations. Control, 1 or 10 μM forskolin or 10 μM (-)-isoproterenol with 10 μM forskolin in the presence of 5 μM (-)-propranolol where indicated. Membranes were prepared 18-48 h after injection of mRNA into the oocytes. Adenylate cyclase activities are expressed as fold elevation over basal activity ± SEM of quadruplicate determinations. The basal values for vehicle-injected oocytes were 0.95 ± 0.05 pmol·mg⁻¹·min⁻¹ for the 1 μM Forsk and 10 μM Forsk + 10 μM Iso panels and 0.58 ± 0.06 pmol·mg⁻¹·min⁻¹ for the 10 μM Forsk panel. The basal values for the mRNA-injected oocyte membrane preparations were 0.62 ± 0.02 pmol·mg⁻¹·min⁻¹ for the 1 μM Forsk and 10 μM Forsk + 10 μM Iso panels and 0.95 ± 0.05 pmol·mg⁻¹·min⁻¹ for the 10 μM Forsk panel.](image)
tropin treatment, became permissive to the passage of fluorescent 6-carboxyfluorescein. If catecholamines act to initiate ion passage or if the basal state of such junctions is ion permeable, the oocyte/follicle cell complex will act as a syncytium. The electrical coupling of the oocyte to the follicle cells within the nascent follicle could explain the isoproterenol-induced hyperpolarization response inasmuch as an electrical response originating in the follicle cell would be transmitted to the oocyte. If the required site of hormone-induced signal initiation was selectively removed during defolliculation, subsequent isoproterenol stimulation would be predicted not to elicit a response. Indeed, we observed that the cell-permeable cAMP analogs 8-bromo-cAMP (Fig. 2), 8-chlorothio-cAMP, and dibutyryl-cAMP (data not shown) do not elicit a hyperpolarization response in the defolliculated oocyte, yet do so in follicle-containing ovarian oocytes. Thus the oocyte membrane itself appears to lack the components necessary for the initiation of the cAMP-dependent hyperpolarization event. The cAMP-mediated event must, therefore, originate in the follicle and then be transmitted to the oocyte membrane by some mechanism, perhaps involving the gap junctional and/or electrical connections that exist between these cell membranes.

We observed that cAMP elevation, on hormone stimulation in whole oocytes, was also dependent on the presence of the follicle. The cAMP content and cyclase activity of follicle-free oocytes and oocyte membranes, respectively, were not stimulated by any concentration of isoproterenol tested (from 0.1 to 1000 μM), although hormone-stimulated adenylate cyclase activity was recovered in membranes prepared from the follicles or follicle-containing ovarian oocytes. Previous reports that oocytes defolliculated by collagenase treatment alone contained forskolin-stimulatable cyclase activity (24) are consistent with our observation that collagenase-treated oocytes showed a forskolin-stimulated increase in cellular cAMP (Table 1). The more complete method of defolliculation presented here does not result in a statistically significant elevation of whole-oocyte cAMP content in response to 100 μM forskolin compared with a threefold elevation in the ovarian oocyte.

The discrepancy between our observation that the forskolin response of defolliculated oocytes is absent or very small and previously published observations that collagenase-defolliculated oocytes contain forskolin-stimulatable cyclase activity could be explained by the presence of small amounts of contaminating follicle membranes adherent to the oocyte membranes defolliculated by collagenase treatment alone. This is most plausible in view of our observations that the specific activity of adenylate cyclase is at least 10 times higher in follicle membranes than in oocyte membranes. Furthermore, in our hands, electron microscopic examination revealed the residual presence of follicle cells in the collagenase-treated oocytes (Fig. 1C) and the absence of adherent follicle cells in the digitonin/collagenase-treated oocytes (Fig. 1D), which essentially lacked forskolin sensitivity (Table 1). We conclude from these data that although the forskolin-sensitive component of adenylate cyclase may be present to a small extent in completely defolliculated oocytes, the isoproterenol-sensitive component is restricted to the follicle cell layer and is not present in the defolliculated oocyte. The data presented in Table 1 and Fig. 2 are also consistent with the hypothesis that the oocyte proper lacks the components necessary for the hyperpolarization and cAMP response to isoproterenol. The possibility that the defolliculation procedure somehow disrupts the oocyte membrane in such a way as to inactivate requisite cyclase components is unlikely in light of the fact that isolated follicle membrane preparations exhibited high levels of hormone-stimulatable cyclase activity inasmuch as they were also exposed to digitonin and collagenase during their isolation. Thus, it is improbable that the brief digitonin exposure acted to inactivate or extract cyclase components from the oocyte membrane proper, because follicle cyclase, being more proximal to the site of treatment, should be expected to be affected first. The concentrations of digitonin used here are many orders of magnitude lower than those typically used to extract cyclase components from membranes. Further, if the high specific activity observed in the follicle membrane cyclase preparation represents a somewhat depressed level of activity owing to digitonin, then the intact follicle activity would be expected to be even higher. Such results would further underscore the critical importance of complete defolliculation. Jordana et al. (24) reported 5'-guanylimidodiphosphate-stimulated activities in ovarian oocytes and follicles isolated by collagenase treatment. Approximately three times the level of adenylate cyclase activity was observed in membranes purified from isolated follicles than from ovarian oocytes.

Having demonstrated that the oocyte membrane proper lacked βAR and forskolin-stimulated adenylate cyclase activity, we microinjected purified mRNA from C6-2B cells. Control vehicle-injected defolliculated oocytes showed no hormone-stimulatable cyclase activity. Elevated adenylate cyclase activity in the presence of isoproterenol alone was observed only in membranes prepared from the C6-2B mRNA-microinjected defolliculated oocytes. However, although statistically significant, this isoproterenol-stimulated activity was quite small. This novel isoproterenol-stimulated adenylate cyclase activity was consistently potentiated by forskolin and blocked by (-)-propranolol to the level of forskolin stimulation alone. In addition, we observed a novel response to forskolin alone in the mRNA-injected oocytes. Thus we conclude that the microinjection into oocytes of heterogeneous mRNA from C6-2B cells has minimally directed the synthesis of the β receptor and the component or components necessary for forskolin-stimulated adenylate cyclase.

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