Cell-free lutropin-dependent desensitization of the lutropin-sensitive adenylate cyclase of pig ovarian follicles is dependent on ATP

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Experiments were conducted to clarify the nucleotide requirements for lutropin (LH)-dependent adenylate cyclase desensitization in a cell-free membrane preparation derived from a thecal-cell-enriched component of preovulatory pig ovarian follicles. The follicular membranes were extensively washed in 2M-urea to remove endogenously bound GTP, and ATP devoid of GTP was utilized. Results conducted in the presence of 60 μ M-GTP and various concentrations of ATP confirm the dependence of LH-stimulated adenylate cyclase activation and desensitization on millimolar concentrations of ATP. In experiments in which adenylate cyclase activation was supported by Mg^{2+} , LH and adenosine 5'-[β,γ -imido]triphosphate, GTP did not support the desensitization response. Moreover, although GTP increased both basal and LH-stimulable adenylate cyclase activities in a concentration-dependent manner, the percentage desensitization was not significantly modified by the presence of 10 nM–10 mM-GTP. These results demonstrate that, even in the presence of exogenous GTP and Mg^{2+} , activation of adenylate cyclase by saturating concentrations of LH in the presence of adenosine 5'-[β,γ -imido]triphosphate is not sufficient to initiate desensitization; millimolar concentrations of ATP are also required for the adenylate cyclase desensitization response.

Hormone–receptor interaction initially activates adenylate cyclase, increasing the rate of cyclic AMP formation. Secondly, the rate of cyclic AMP formation is often decreased (see review by Perkins *et al.*, 1982). In several such systems, including the preovulatory ovarian follicle, this decrease in the rate of cyclic AMP synthesis is not (initially) accompanied by a decrease in receptor numbers, nor is it due to receptor occupancy (Bockaert *et al.*, 1976; Ezra & Salomon, 1980).

Adenylate cyclase activation and subsequent desensitization in preovulatory ovarian follicles can be initiated *in vivo* (Hunzicker-Dunn & Birnbaumer, 1976*a,b*) in response to the preovulatory gonadotropin surge as well as *in vitro* by incubating intact follicles with LH (Lamprecht *et al.*, 1973; Hunzicker-Dunn & Birnbaumer, 1981). These responses of the follicular adenylate cyclase

to LH can also be demonstrated in a cell-free follicular-membrane preparation (Bockaert *et al.*, 1976; Hunzicker-Dunn *et al.*, 1979). To determine the molecular basis for adenylate cyclase desensitization, the molecular requirements needed to achieve cell-free desensitization have been evaluated. With a membrane preparation derived from preovulatory pig ovarian follicles, LH-dependent desensitization of adenylate cyclase has been shown to be a time-dependent reaction, albeit slower than activation, dependent on the concentrations of LH, Mg^{2+} and ATP (Bockaert *et al.*, 1976). This requirement for ATP could not be fulfilled by the non-hydrolysable ATP analogue p[NH]ppA, even though this compound supported adenylate cyclase activation (Bockaert *et al.*, 1976). LH-dependent desensitization also did not proceed when 10 μ M-GTP was substituted for 1 mM-ATP during the 'desensitization phase' of the incubation. However, a 'secondary role' for GTP after adenylate cyclase activation by millimolar ATP concentrations could not be excluded from these studies. The presence of GTP in the cell-free membrane system studied by Bockaert *et al.*

Abbreviations used: LH, lutropin; p[NH]ppA, adenosine 5'-[β,γ -imido]triphosphate; p[NH]ppG, guanosine 5'-[β,γ -imido]triphosphate.

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(1976), either supplied as a contaminant of ATP or elsewhere, is evidenced by the demonstration of hormone-stimulated adenylate cyclase activity without the addition of exogenous GTP. Thus, in the cell-free membrane preparation derived from preovulatory pig follicles, adenylate cyclase activation may be a prerequisite for adenylate cyclase desensitization, the latter response potentially mediated by GTP.

The nucleotide requirements for adenylate cyclase desensitization have been studied in only a few other systems. Ezra & Salomon (1981) showed that LH-dependent adenylate cyclase desensitization to LH in a cell-free membrane preparation derived from hormone-primed immature rat ovaries required GTP and Mg^{2+} , and not ATP. They concluded that catalytic cyclase activity was probably not a prerequisite for desensitization in their membrane preparation. In contrast with the rat ovary system, cell-free adenylate cyclase desensitization to isoprenaline in rat kidney cells and to glucagon in rat liver required Mg^{2+} , GTP and ATP (Anderson & Jaworski, 1979; Iyengar *et al.*, 1980). Hormone-dependent adenylate cyclase desensitization in these systems was not achieved when GTP or ATP was omitted. A prerequisite for adenylate cyclase activation, potentially as the basis for the ATP requirement, also cannot be ruled out in these latter studies.

The purpose of the experiments described below was to clarify the nucleotide requirements for LH-dependent adenylate cyclase desensitization of the LH-activated cyclase in a cell-free membrane preparation derived from preovulatory pig ovarian follicles. Results conducted in the presence of a saturating concentration of GTP and various concentrations of ATP confirm the dependence of LH-dependent adenylate cyclase desensitization on millimolar concentrations of ATP. Activation of adenylate cyclase by GTP, Mg^{2+} and a saturating concentration of LH in the presence of p[NH]ppA is not sufficient to induce adenylate cyclase desensitization; millimolar concentrations of ATP are still required to decrease the rate of cyclic AMP synthesis.

Materials and methods

LH (NIH-LH-B9) was generously given by the National Institutes of Health, Bethesda, MD, U.S.A. p[NH]ppA and p[NH]ppG were both purchased from Boehringer-Mannheim, Indianapolis, IN, U.S.A. GTP-free ATP (prepared by phosphorylation of adenosine), cyclic AMP, GTP and Bistris {2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol} were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Phosphocreatine and creatine kinase were

from Calbiochem, La Jolla, CA, U.S.A. Ultra-pure urea was purchased from Schwartz/Mann, Spring Valley, NY, U.S.A. Dowex AG 50-X4 (200–400 mesh, H^+ form) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Aluminium oxide (90 active, neutral) was from E. Merck Chemicals, Emarstadt, Germany.

Radiochemicals

[α - ^{32}P]ATP (ammonium salt; 20–50 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Cyclic [2,8- 3H]AMP (ammonium salt; 30–40 Ci/mmol) was purchased from Amersham Radiochemicals, Arlington Heights, IL, U.S.A. All radiochemicals were used without further purification.

Preparation of ovarian-follicle membranes

Pig ovaries, obtained from a local slaughterhouse, were placed in iced Krebs–Ringer bicarbonate buffer (Cohen, 1951) for transport to the laboratory. The follicular wall (predominantly consisting of thecal cells) from ovarian follicles greater than 6 mm in diameter was collected, and an adenylate cyclase-enriched membrane fraction was isolated from these cells by previously described methods with some alterations (Bockaert *et al.*, 1976). An initial centrifugation step (1200g for 10 min) was included to remove the heavier cell components. The supernatant was then spun at 15000g for 15 min, and the pellet obtained was resuspended in 10 vol. of 10 mM-Tris/HCl containing 2 M-urea (pH 7.1–7.4) and left at room temperature for 5 min. The membranes were extensively washed by three consecutive centrifugations each at 15000g for 15 min, the pellets being resuspended each time in 10 mM-Tris/HCl (pH 7.1–7.4). The final pellet was resuspended in 27% (w/w) sucrose/10 mM-Tris/HCl (pH 7.1), divided into batches and quick-frozen in an acetone/solid- CO_2 bath, and stored at $-70^\circ C$ for not more than 2 months. All centrifugations were at $4^\circ C$.

Adenylate cyclase assay

Incubation and assay of membrane particles for adenylate cyclase activity and desensitization was performed in a two-stage reaction at $30^\circ C$ by previously described methods (Bockaert *et al.*, 1976), except for modifications pertaining to the particular experimental procedures described in each Figure legend. The two-stage reaction consisted of an initial 35 min incubation phase followed by a 5 min assay phase. The incubations were initiated by the addition of 20 μl of incubation medium/regeneration system and 10 μl of nucleotide. Components of the incubation medium/regeneration system included 0.4 mM-EDTA, 1.0 mM-EGTA, 25 mM-Bistris, 5 mM- $MgCl_2$,

20mM-phosphocreatine, 0.2mg of creatine kinase/ml, 1mM-cyclic [^3H]AMP (10000c.p.m.), and either LH (desensitized) or bovine serum albumin (full activity or basal) at 15 $\mu\text{g}/\text{ml}$. GTP-free ATP and/or GTP, at concentrations appropriate for each experiment (see the Figure legends), was contained in a 10 μl sample added to each sample tube before incubation. At the end of the incubation phase, the 5min assay phase was initiated by the addition of 10 μl of either LH or bovine serum albumin at 15 $\mu\text{g}/\text{ml}$, and [$\alpha\text{-}^{32}\text{P}$]ATP (6 $\times$ 10 6 c.p.m./tube). Final concentrations of components are stated and are based on 60 μl final assay volumes.

Calculation of the percentage of LH-mediated desensitization of the cyclase system to subsequent LH-stimulated cyclic AMP-production (%D) was performed by the equation:

$$\%D = 100\% \times \left[1 - \frac{(\text{LH/LH}) - (\text{BSA/BSA})}{(\text{BSA/LH}) - (\text{BSA/BSA})} \right]$$

where LH/LH is the rate of LH-stimulated cyclic AMP production (pmol of cyclic AMP/min per mg of protein) in samples preincubated with LH (desensitized); BSA/LH is the rate of LH-stimulated cyclic AMP production in samples preincubated with bovine serum albumin (full activity), and BSA/BSA is cyclic AMP production in samples containing bovine serum albumin only in both incubation and assay phase (basal).

Results

Effect of ATP concentration on adenylate cyclase activity and desensitization

Previous studies demonstrated the dependence of cell-free adenylate cyclase activity on the ATP concentration (Bockaert *et al.*, 1976). However, it was not possible to determine whether or not GTP, possibly introduced as a contaminant of the exogenous ATP preparation, was influencing the rate of cyclic AMP production in the LH-stimulated or desensitized cell-free adenylate cyclase system. Therefore experiments were conducted to evaluate the dependence of cell-free adenylate cyclase activity and desensitization on the ATP concentration, by using an ATP preparation free of exogenous GTP.

It was first necessary to determine at what GTP-free ATP concentration maximal cyclase activity occurred, and to show the relationship between the ATP concentration and cyclase activity. Fig. 1 clearly shows that the LH-stimulated cyclic AMP production by the cell-free adenylate cyclase is dependent on the ATP concentration. The maximal rate of cyclic AMP production for basal (BSA/BSA) and fully stimulated (BSA/LH) cyclase

Fig. 1. Effect of ATP concentration on adenylate cyclase activity and desensitization

LH-mediated stimulation and subsequent desensitization to further LH stimulation of the adenylate cyclase system was tested with GTP-free ATP at the indicated concentrations. ATP was added to the incubation phase of the reaction as described in the Materials and methods section. Final ATP concentrations (based on the 60 μl total volume reaction) are indicated. Δ , LH-stimulated cyclic AMP production: incubation phase with bovine serum albumin, assay phase with LH (BSA/LH; full activity). $\square$, LH-stimulated cyclic AMP production: incubation phase with LH, assay phase with LH (LH/LH; desensitized). $\circ$, Basal rate of cyclic AMP production: incubation phase with bovine serum albumin, assay with bovine serum albumin (BSA/BSA; basal). Points represent individual incubations. Regression analysis, by using an equation of the form $y = a + bx + cx^2$, was used to generate the curves shown in this Figure. Coefficients of determination (R^2), of 0.955 (LH/LH), 0.964 (BSA/LH), and 0.960 (BSA/BSA), were obtained for each curve shown (Armitage, 1971).

occurred at about 1mM-ATP. This maximal stimulation at 1mM-ATP, with an ATP preparation free of exogenous GTP, was consistent with previously reported results where the possibility of GTP introduction, as a contaminant of added ATP, was not addressed (Bockaert *et al.*, 1976). Cyclic AMP production in samples desensitized to further LH stimulation (LH/LH) also reached an observed maximum at 1mM-ATP (Fig. 1). From these data, a % D of about 36% can be calculated (at 1mM-ATP). The observed %D obtained in this cell-free cyclase system agreed well with %D values previously obtained for desensitized cell-free adenylate cyclase systems (also determined at

1 mM-ATP), that may have contained GTP as part of the exogenous ATP preparation (Bockaert *et al.*, 1976).

It was also decided to examine the kinetic relationship between ATP concentration and cell-free adenylate cyclase activity and desensitization. Application of kinetic analysis to the data shown in Fig. 1 gave the Eadie-Hofstee plots shown in Fig. 2. ATP concentrations of 0.10–1.00 mM were used in the construction of these plots. The slopes obtained from these lines were used to estimate an apparent K_m (mM-ATP) for this cell-free preparation for each of the three sets: K_m (LH/LH) = 0.15 ± 0.013 , K_m (BSA/LH) = 0.16 ± 0.016 and K_m (BSA/BSA) = 0.11 ± 0.019 . The apparent K_m values obtained thus were not significantly different from each other ($P \leq 0.05$). These results suggest that differences in the affinity of the cell-free adenylate cyclase for the ATP substrate, based on the presence of LH (at a concentration of LH up to $30 \mu\text{g/ml}$) cannot be detected. An apparent $V_{\max}$ (pmol of cyclic AMP/min per mg) for each of the three sets was obtained from the intercepts of the respective regression lines, as follows: $V_{\max}$ (LH/LH) = 28.4 ± 0.93 , $V_{\max}$ (BSA/LH) = $39.5 \pm$

1.5 and $V_{\max}$ (BSA/BSA) = 9.2 ± 0.52 . The apparent $V_{\max}$ values obtained thus were statistically different from one another ($P \leq 0.05$). These apparent $V_{\max}$ values, taken in conjunction with the observed apparent K_m values, suggest that the cell-free adenylate cyclase preparation used in this study can be tentatively classed as a $V_{\max}$ -type enzyme system, when ATP is the substrate (Segel, 1975).

Having demonstrated the dependence of the rate of cyclic AMP production by the LH-stimulated cell-free adenylate cyclase on GTP-free ATP concentrations, it was then necessary to determine the effect of ATP concentration on cell-free adenylate cyclase desensitization. To this end LH/LH, BSA/LH and BSA/BSA sample sets were first incubated at several GTP-free ATP concentrations (see Fig. 3), followed by the addition of the amount of ATP required to adjust each sample to 2.0 mM-ATP at the start of the assay phase. This protocol makes the observed rate of cyclic AMP production for each sample consistent with regard to assay-phase ATP concentrations. Thus, if LH-mediated desensitization is dependent on the ATP concentration, then desensitization should occur as a function of the amount of ATP present only during the incubation phase. As shown by the data in Fig. 3 (lower panel), the rate of cyclic AMP production in samples incubated with LH (LH/LH) decreased as the GTP-free ATP concentration of these samples increased. No significant decrease, with increasing ATP concentration of the incubation medium, was observed for cell-free adenylate cyclase samples first incubated with bovine serum albumin and then stimulated by LH addition at the start of assay phase (BSA/LH). Samples representing the basal rate of cyclic AMP production by the cell-free adenylate cyclase in the total absence of exogenous LH (BSA/BSA) showed a slight but statistically significant ($P \leq 0.05$) decline in the rate of cyclic AMP production with increasing ATP concentration in the incubation medium. This is thought to reflect minimal desensitization caused by a small amount of endogenous LH present as part of the cell-free adenylate cyclase preparation. Taken together, the results obtained for all three adenylate cyclase sample sets provide evidence that desensitization, up to a limiting extent, is (in part) dependent on the ATP concentration in the cell-free adenylate cyclase system. The data shown in Fig. 3 more directly demonstrate this observation. Here, the % D (calculated as indicated in the Materials and methods section) steadily increases as the ATP concentration of the incubation medium increases.

The ATP analogue p[NH]ppA has previously been shown to be a suitable substrate for the cell-free adenylate cyclase system, resulting in the

Fig. 2. Eadie-Hofstee treatment of data from Fig. 1. Individual results from Fig. 1 representing ATP concentrations from 0.10–1.00 mM-ATP were transformed as indicated on the abscissa of this Figure. $\circ$, BSA/LH; $\triangle$, LH/LH; $\bullet$, BSA/BSA; V_0 , velocity at $[S]$, concentration of ATP. Best-fit lines were generated by using linear regression for each set of data (Segel, 1975; Armitage, 1971).

Fig. 3. Dependence of adenylate cyclase desensitization on ATP concentration

Lower panel: the incubation phase was conducted in the presence of the indicated ATP concentrations, 10 μ M-GTP and bovine serum albumin (●, Δ) or LH ($\square$). The assay phase was initiated by the addition of an amount of ATP appropriate to give total ATP concentrations in each tube of 2.0 mM as described in the Materials and methods section. Linear-regression analysis of BSA/LH data shows regression not to be significant, and therefore the line traversing these points is drawn through the arithmetic mean of all BSA/LH data shown in the Figure. LH/LH data were fitted by third-order regression. Regression of BSA/BSA data was found to be significant ($P \leq 0.05$). Upper panel: the curve representing the effect of increasing ATP concentrations on %D was obtained by third-order regression ($R^2 = 0.942$) on the %D values ($\circ$) as derived from the data in the lower panel. These %D values were calculated as described in the Materials and methods section, by using the observed LH/LH value at the ATP concentration of interest, the mean for all BSA/LH observations, and the BSA/BSA value estimated from the regression of BSA/BSA data, at the ATP concentration of interest.

production of cyclic AMP (Bockaert *et al.*, 1976). Furthermore, it has also been demonstrated that cell-free adenylate cyclase desensitization is independent of p[NH]ppA concentration (Bockaert *et al.*, 1976). Therefore, we reasoned that by adding GTP-free ATP and p[NH]ppA in a ratio such that the total nucleotide concentration of the incubation medium is maintained constant at 1.25 mM, the dependence of the rate of cyclic AMP production on total adenine nucleotide concentration in the non-desensitized cell-free adenylate

Fig. 4. Effect of p[NH]ppA and ATP on adenylate cyclase activity and desensitization

Lower panel: LH-mediated activity and desensitization of the cyclase system was measured in samples containing ATP and p[NH]ppA at respective concentrations (mM) of 1.25/0, 1.00/0.25, 0.75/0.50, 0.50/0.75, 0.25/1.00 and 0/1.25 as indicated, to maintain a total ATP + p[NH]ppA concentration of 1.25 mM per sample. ATP and p[NH]ppA were added in a single volume of 10 μ l at the beginning of the incubation phase; otherwise, incubation and assay phases were performed as described in the Materials and methods section, without the addition of unlabelled ATP to the assay phase. GTP (60 μ M) was present throughout. Regression on BSA/LH (Δ , not significant at $P \leq 0.05$) and BSA/BSA (●, significant at $P \leq 0.05$) data, and curve-fitting on LH/LH data ($R^2 = 0.990$), were performed as stated for Fig. 3. The line drawn traversing BSA/LH data represents the arithmetic mean of all BSA/LH observations shown. Upper panel: the curve representing the effect of increasing ATP concentrations and decreasing p[NH]ppA concentration at each ratio on %D ($\circ$) was obtained as stated for Fig. 3 (upper panel) ($R^2 = 0.992$).

cyclase system (BSA/LH, BSA/BSA) would be eliminated. At the same time, the dependence of the extent of desensitization on the ATP concentration in samples incubated in the presence of LH (LH/LH) should become apparent. This approach was taken to corroborate the results shown in Fig. 3 and to minimize the possible variation in total adenine nucleotide concentration in each sample tube resulting from the second ATP addition used in generating results shown in Fig. 3.

As shown in Fig. 4, the rates of cyclic AMP production versus ATP concentration for BSA/LH, LH/LH, and BSA/BSA sample sets followed virtually the same trends as those ob-

served for the analogous samples shown in Fig. 3. That p[NH]ppA functions as a suitable substrate for adenylate cyclase in the absence of unlabelled ATP was also demonstrated (Fig. 4, 0mM-ATP, 1.25mM-p[NH]ppA). These data provide further evidence that the extent of LH-mediated desensitization in these cell-free adenylate cyclase systems is in part dependent on the ATP concentration.

Effect of GTP concentration on adenylate cyclase activity and desensitization

It has been demonstrated that hormone-stimulated adenylate cyclase activation exhibits an absolute requirement for GTP (as reviewed by Limbird, 1981). To assess the possible effects of exogenous GTP on the activity of the cell-free adenylate cyclase preparation used in our laboratory, a profile of the rate of cyclic AMP production by the cyclase system over a range of GTP concentrations was obtained. Samples containing bovine serum albumin were incubated for 35 min with the indicated concentrations of GTP, in the presence of 1.25mM-ATP. The assay phase was initiated by the addition of radiolabelled ATP in the presence of bovine serum albumin or LH. Results presented in Fig. 5 demonstrate that

Fig. 5. *Effect of GTP concentration on adenylate cyclase activity*

The LH-stimulated production of cyclic AMP was evaluated at the indicated GTP concentrations. BSA/LH (Δ) and BSA/BSA ($\circ$) sample sets, at each of the GTP concentrations of interest, were incubated as described in the Materials and methods section in the presence of 1.25mM-ATP. The assay phase was carried out as in the Materials and methods section.

exogenous GTP increased the rate of cyclic AMP production for both LH-stimulated (BSA/LH) and basal (BSA/BSA) cyclase sample sets, over the GTP concentration range 0.1 μ M–0.1mM. With exogenous GTP concentrations above 0.1mM and up to 10mM, rates of cyclic AMP production fell dramatically. These observations are consistent with results reported for other systems, where similar dependencies of adenylate cyclase activity on GTP concentrations have been described, with a $K_{0.5}$ for GTP of 0.1–0.01 μ M (as reviewed by Limbird, 1981). Below concentrations of 0.1 μ M-GTP, the rate of cyclic AMP production did not vary with the GTP concentration. We expect that the high rate of cyclic AMP production observed with GTP concentrations below 0.1 μ M is due to the contribution of endogenously bound GTP in the membrane preparation, not removed by the 2M-urea-wash step (see the Materials and methods section). From the increase in cyclic AMP production by GTP concentrations only above 0.1 μ M, it can be predicted that GTP is present in the membrane preparation at concentrations below 1 μ M.

Others have suggested that the influence of GTP on adenylate cyclase activity is modulated by the rate of hydrolysis of GTP to GDP + P_i, with the hydrolysis of GTP being reflected as a concomitant decrease in the rate of cyclic AMP production (as reviewed by Limbird, 1981). It was therefore decided to examine the rate of cyclic AMP production in cell-free adenylate cyclase incubations, where the non-hydrolysable GTP analogue p[NH]ppG had been substituted for GTP, at concentrations of 0–10mM added p[NH]ppG. Results in Fig. 6 show the dependence of cyclic AMP production on the concentration of p[NH]ppG. The LH-stimulated adenylate cyclase activity (BSA/LH) increased significantly to a maximum with 0.1mM-p[NH]ppG, a concentration essentially the same as that observed for adenylate cyclase incubations containing GTP. And, as with cyclase incubations containing GTP, the rate of cyclic AMP production declined rapidly with p[NH]ppG concentrations above 0.1mM. Basal (BSA/BSA) rates of cyclic AMP production also increased dramatically with increasing p[NH]ppG concentration to a maximum at 0.1mM. Basal rates of cyclic AMP production at 0.1mM-p[NH]ppG approached the rates observed for adenylate cyclase activity in the presence of hormone (BSA/LH). These observations are consistent with, and further support, results that demonstrate the dependence of the rate of cyclic AMP production on GTP concentration (see Fig. 5).

Having demonstrated that adenylate cyclase activation was dependent on GTP, experiments were conducted to evaluate the role of GTP in

Fig. 6. Effect of $p[\text{NH}]ppG$ concentration on adenylate cyclase activity

The GTP analogue $p[\text{NH}]ppG$ was added to each sample tube before the incubation phase, to give the concentrations indicated. Incubation and assay phases were performed as stated in Fig. 5. No exogenous GTP was present. Sample sets were BSA/LH (Δ) and BSA/BSA ($\circ$).

hormone-induced adenylate cyclase desensitization. Previous studies by Bockaert *et al.* (1976) demonstrated that desensitization did not proceed in the presence of Mg^{2+} and LH when $10 \mu\text{M}$ -GTP was substituted for 1 mM -ATP. We have confirmed this finding in Fig. 3, showing that there is no decrease in the rate of cyclic AMP production in the presence of Mg^{2+} , $60 \mu\text{M}$ -GTP and a saturating LH concentration when ATP is omitted from the 'desensitization phase' of the incubation.

Experiments reported in Fig. 4 directly address the possibility that the millimolar-ATP requirement for adenylate cyclase desensitization demonstrated in Fig. 3 derives from the ability of ATP to serve as a substrate for adenylate cyclase and as such, in the presence of GTP, Mg^{2+} and LH, to allow adenylate cyclase activation, and not from a direct requirement for ATP in the desensitization response. Results showed that, after the activation of adenylate cyclase by a saturating concentration of LH, with Mg - $p[\text{NH}]ppA$ as a substrate, $60 \mu\text{M}$ -GTP did not give any decrease in the rate of cyclic AMP production (Fig. 4). Under these same conditions, LH-dependent adenylate cyclase desensitization was not achieved with GTP concen-

Fig. 7. Effect of GTP on adenylate cyclase desensitization

The desensitization of the cyclase system to subsequent LH-stimulated cyclic AMP production was measured in LH/LH, BSA/LH, and BSA/BSA sample sets (six samples/set) in both the absence (GTP^-) and the presence (GTP^+) of 0.1 mM -GTP. ATP (1.00 mM) was present throughout the incubation and assay phases. Error bars represent $\pm \text{s.d.}$

trations in the range 1 nM – 1 mM during the 'desensitization phase' of the reaction (results not shown). Adenylate cyclase desensitization occurs only when ATP is included.

Lastly, experiments were conducted to determine whether LH-induced adenylate cyclase desensitization, occurring in the presence of MgATP , could be enhanced by exogenous GTP. Incubations were conducted in the presence of 1.0 mM -ATP both without (GTP^-) and with (GTP^+) exogenous GTP, the latter in the range 10 nM – 10 mM . A profile equivalent to that represented in Fig. 5 was obtained, where GTP concentrations of $0.1 \mu\text{M}$ – 0.1 mM increased the rates of cyclic AMP production for all parameters evaluated (BSA/BSA, BSA/LH, LH/LH), whereas GTP concentrations above 0.1 mM produced a drastic decline in the rates of cyclic AMP production (results not shown). As shown in Fig. 7, the addition of 0.1 mM -GTP to the incubation medium resulted in a significant increase in the overall rate of cyclic AMP production for LH/LH, BSA/LH and BSA/BSA samples over equivalent controls lacking exogenous GTP. The calculated %D values for both the GTP^+ and GTP^- samples were 40.7% and 35.7% respectively. To determine

whether or not the addition of GTP resulted in a significant increase in %D over GTP⁻ controls, two-way analysis of variance (six observations/cell) was performed on the natural logarithms of the fold stimulation over basal values derived from these data. By this analysis, the %D values of 40.7% and 35.7% were both found to be significant, as was the influence of GTP in increasing the overall rate of cyclic AMP production for each of the LH/LH and BSA/LH sample sets over their respective GTP⁻ control $P \leq 0.05$. However, the difference in %D observed for GTP⁺ and GTP⁻ samples (40.7% and 35.7% respectively) was not significantly different ($P \leq 0.05$) at any concentration of GTP evaluated. From this analysis it is suggested that, although the presence of GTP in the cell-free cyclase system may result in an increase in the observed rate of LH-stimulated cyclic AMP production, its influence on the rate of desensitization (%D) appears minimal under these conditions.

Discussion

The purpose of this work was to clarify the nucleotide requirements of LH-dependent adenylate cyclase desensitization in a cell-free membrane preparation derived from a thecal-cell-enriched component of preovulatory pig ovarian follicles. To this end, membranes were extensively washed in 2M-urea to remove endogenously bound GTP, and an ATP preparation synthesized from adenosine and devoid of contaminating GTP was used.

Both ATP and p[NH]ppA, in the presence of Mg²⁺, functioned as equivalent substrates in terms of the rates of cyclic AMP production for LH-stimulated activation of the adenylate cyclase present in the cell-free membrane preparation. GTP markedly increased both basal and LH-stimulated adenylate cyclase activities, demonstrating a maximal effectiveness at 0.1 mM-GTP. We were, however, unable to demonstrate an absolute dependence of adenylate cyclase activation on exogenous GTP, since both basal and LH-stimulated activities were measurable in the absence of added GTP. It therefore may be concluded that some GTP (predicted to be less than 1 μ M) remained associated with the membrane particles, despite our attempts to dislodge it.

In spite of the demonstrable effect of GTP on adenylate cyclase activation, the extent of LH-stimulated adenylate cyclase desensitization was not affected by GTP. GTP was ineffective in either promoting or modifying the extent of adenylate cyclase desensitization, under conditions where catalytic adenylate cyclase activity was raised by LH and Mg²⁺ with either p[NH]ppA or ATP as cyclase substrates. Although we cannot

experimentally rule out the possibility that sufficient GTP was endogenously present to meet the requirements for this nucleotide in mediating desensitization, since the maximally effective concentration of endogenous GTP must be of the order of 0.1 μ M, a $K_{0.5}$ at least one to two orders of magnitude lower would be required. This is an extremely low concentration of GTP in a biological system.

The basis for the requirement for ATP in adenylate cyclase desensitization is not known. Since ATP is required for activation, it is difficult to assess whether adenylate cyclase activation is indeed a prerequisite for the desensitization response. The requirement for millimolar concentrations of ATP would suggest a substrate role, especially since an equivalent ATP-dependency curve is derived for adenylate cyclase activation. From the inability of p[NH]ppA to support desensitization (Bockaert *et al.*, 1976) and the ability of a phosphoprotein phosphatase-containing extract of pig follicles to reverse, in a Mn²⁺-dependent manner, LH-induced desensitization of the LH-sensitive adenylate cyclase of pig Graafian follicles (Hunzicker-Dunn *et al.*, 1979), it can be concluded that uncoupling of the hormone-receptor complex from the regulatory proteins occurs as a result of a hormone-dependent phosphorylation reaction, possibly of the receptor. In those systems where adenylate cyclase desensitization is not linked to receptor down-regulation, it is generally agreed that the initial phase of hormone-dependent adenylate cyclase desensitization involves a decreased interaction of the hormone-occupied receptor with the regulatory proteins (Iyengar *et al.*, 1981; Dix *et al.*, 1982; Rapoport *et al.*, 1982; Kassis & Fishman, 1982). The functional capacities of neither the regulatory proteins nor the catalytic moieties of adenylate cyclase appear to be compromised (Sen *et al.*, 1979; Pike & Lefkowitz, 1980; Iyengar *et al.*, 1981; Dix *et al.*, 1982; Kassis & Fishman, 1982). Subsequent studies have in fact demonstrated that catecholamine-induced desensitization of adenylate cyclase in turkey erythrocytes is associated with β -receptor phosphorylation (Stadel *et al.*, 1983). However, evidence for a functional uncoupling of the phosphorylated receptor and cyclase regulatory proteins was not provided. Possibly ATP supplies two functions: one, as substrate for adenylate cyclase in the activation reaction and two, as the mediator of desensitization. Further studies are required to clarify the mechanism(s) by which ATP regulates adenylate cyclase desensitization.

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