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PHOTO-INDUCED INACTIVATION AND UNCOUPLING OF GONADOTROPIN RECEPTORS IN RAT OVARIAN PLASMA MEMBRANE

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Summary

Suspensions of purified rat ovarian plasma membranes were irradiated by high-intensity light in the cold. This treatment gradually reduced the ability of the membrane receptor to bind ^{125}I-labeled human chorionic gonadotropin and the ability of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) to respond to luteinizing hormone, follicle-stimulating hormone, human chorionic gonadotropin and prostaglandin E_2. In contrast adenylate cyclase activity stimulated by NaF or guanosine 5′-(β, γ-imido)triphosphate (p(NH)ppG) was significantly more resistant to irradiation. Human chorionic gonadotropin protected the binding site from light-induced damage, but not the ability of the hormone to activate adenylate cyclase. Irradiation destroyed close to 50% of unoccupied guanosine nucleotide binding sites but apparently did not induce massive covalent binding of nucleotides to membrane components. It is suggested that high-intensity light induces damage to two separate sites in the adenylate cyclase system. One affects hormone binding and is presumably associated with the hormone receptor, the second interferes with coupling but at a step proximal to regulation of adenylate cyclase by GTP binding protein.

Introduction

Occupation of some cell membrane receptors by specific hormones leads to stimulation of adenylate cyclase. This complex transmembrane system is

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Abbreviations: FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; p(NH)ppA, adenosine 5′-(β,γ-imido)triphosphate; p(NH)ppG, guanosine 5′-(β,γ-imido)triphosphate; GF, guanosine nucleotide regulatory component.

known to consist of several components: the receptor which binds the hormone; the enzyme which catalyses the formation of cyclic 3′,5′-AMP from ATP, and regulatory components [1] which specifically interact with GTP and are essential for NaF stimulation [2—4]. Little is known about the molecular properties and special orientation of these components in the membrane. However, catalytic activity has been suggested to be under tight control by nucleotide regulatory proteins [5—8], whereas the activity of these regulatory proteins has been postulated to be under receptor control [9].

Ovarian adenylate cyclase is stimulated by luteinizing hormone (LH), and human chorionic gonadotropin (hCG) apparently via a common receptor site [10]. This enzyme system is of key importance in the regulation of ovulation and steroidogenesis [11,12]. The mechanism by which the gonadotropic hormones control ovarian adenylate cyclase is therefore of great interest. One approach to study the mode of interaction of the components of this enzyme system would be to modify them in a way which will selectively affect their function. Study of properties of such modified enzyme preparations may yield new information about their functional relationship. Since protein-protein interactions as well as protein-nucleotide interactions are involved in adenylate cyclase activity, we thought to apply rather nonspecific means.

High-intensity light irradiation has been shown to cross-link proteins with proteins and proteins with nucleic acids [13—18]. Such techniques were applied in the study of the interactions of DNA with histone [17], histone with ATP [18], and ribonuclease with the specific inhibitors, cytidine 2′(3′),5′-diphosphate and uridine 2′(3′),5′-diphosphate [13,27].

As shown in this study high-intensity light affects adenylate cyclase activity and proved to be an interesting tool to functionally uncouple the receptor from the enzyme. Furthermore, the destructive effect of light on the hormone receptor was prevented by the hormone, a finding suggestive of conformational changes induced in the receptor by the bound hormone molecule.

Materials and Methods

Materials

[α-^{32}P]ATP, -cyclic 3′5′[8-^{3}H]AMP, Na^{125}I carrier free, and [8-^{3}H]guanosine 5′-(β,γ-imido)triphosphate were obtained from the Radiochemical Center, Amersham, U.K. ATP, cyclic 3′5′-AMP, dithiothreitol, sodium fluoride, bovine serum albumin, phosphocreatine, creatine phosphokinase and lactoperoxidase were products of Sigma Chemical Co. Guanosine 5′-(β,γ-imido)triphosphate (p(NH)ppG) and adenosine 5′-(β,γ-imido)triphosphate (p(NH)ppA) were obtained from the International Chemical Co. Ovine luteinizing hormone (LH) (NIH-LH-S18; specific potency 1.03 × NIH-LH-SI by ovarian ascorbic acid depletion test) and rat follicle-stimulating hormone (FSH) (FSH-I-3 biological potency 150 × NIH-FSH-S1) were kindly supplied by the U.S. National Institutes of Health.

Human chorionic gonadotropin (hCG, Pregnyl N.V.) was obtained from Organon, Oss, Holland. Highly purified hCG (13 400 I.U./mg) was a product of Serono, Rome, Italy.

Prostaglandin E_2 was supplied by courtesy of Dr. J. Pike of the Upjohn Co., Kalamazoo, MI, U.S.A.

Preparation of ovarian plasma membranes

Plasma membranes from rat ovaries were purified as previously described by Mintz et al. [19].

Adenylate cyclase assay

Activity was assayed by measuring the formation of cyclic [^{32}p]AMP from [α-^{32}P]ATP. Assay conditions were described previously [19,20]. Cyclic [^{32}P] AMP was isolated according to the method of Salomon et al. [21]. The reaction was initiated by the addition of plasma membranes (4 μg per assay, unless otherwise stated). The reaction was terminated after 10 min incubation at 30° C. The activity ratio was defined as the adenylate cyclase activity in the presence of hormone divided by the adenylate cyclase activity in its absence.

Iodination of hCG

Highly purified hCG was iodinated with ^{125}I by the lactoperoxidase method described by Miyachi et al. [22].

The biological activity of the iodinated ^{125}I-labeled hCG was determined by its ability to stimulate hCG-sensitive adenylate cyclase as compared to a standard curve obtained with known concentrations of unlabeled hCG.

^{125}I-labeled hCG binding

Binding of ^{125}I-labeled hCG to membrane preparations was measured by Millipore filtration according to Rubalcava and Rodbell [23]. The incubating mixture (50 μl final volume) contained 25 mM Tris/maleate, pH 7.0, 0.6% bovine serum albumin and ^{125}I-labeled hCG ($0.4—2 \cdot 10^5$ cpm). The binding reaction was initiated by the addition of plasma membranes (10 μg per assay) and terminated after 20 min incubation at 30° C by dilution with 5 ml 2.5% bovine serum albumim buffered in 25 mM Tris/acetate, pH 7.6.

The samples were filtered through Millipore filters, washed once with 5 ml 1% bovine serum albumim buffered in 25 mM Tris/acetate, pH 7.6, and twice with 2 ml of the same solution.

Nonspecific binding was determined in the presence of excess unlabeled 400—800 nM hCG (Pregnyl) as indicated in each experiment. In addition, blanks without plasma membranes were included. These controls gave similar values that did not exceed 0.5—1% of the total ^{125}I-labeled hCG added to the reaction mixture, and 5—10% of the total ^{125}I-labeled hCG bound. (Unless otherwise indicated these values were substracted from the total binding.)

[8-^{3}H]p(NH)ppG binding assay

Binding of [8-^{3}H]p(NH)ppG to plasma membrane was measured by the filtration technique previously described for the binding of [8-^{3}H]GTP and [8-^{3}H]p(NH)ppG to hepatic plasma membranes [24]. The reaction was initiated by the addition of 10 μg plasma membranes and was terminated by filtration after 15 min of incubation at 30° C.

Irradiation with high-intensity light

Plasma membrane suspensions in a pyrex test tube (16 mm outer diameter and 12 mm inner diameter) were immersed in a water/ice bath (4° C) 5 cm from

the center of a Hanovia, 450 W, high-pressure mercury lamp. The lamp was cooled by circulating water. The test tube also served as a filter to exclude light of $\lambda > 300$ nm. Samples of the membrane suspension were withdrawn at various times and transferred into a dark compartment kept at 4°C. Residual activities of ^{125}I-labeled hCG binding, [8-^{3}H]p(NH)ppG binding and adenylate cyclase were assayed within 30 min. These activities were unchanged for several hours if the membrane suspensions were kept at 4°C.

The effect of high-intensity light on the stability of the complex of ^{125}I-labeled hCG or [8-^{3}H]p(NH)ppG with their respective binding sites (preformed complex) was also determined. In these experiments membranes were first incubated with the radioactive ligand under standard conditions. Subsequently the suspensions were exposed to light and samples (50 µl) were withdrawn at various times to determine the amount of bound ligand.

Dissociation and reassociation of the receptor-hormone complex and the ability to activate adenylate cyclase

Dissociation of hCG from plasma membranes was performed according to the procedure described by Amir and Salomon [25] according to the following scheme:

Stage 1. Plasma membranes (0.2 mg/ml) were incubated with ^{125}I-labeled hCG at 30°C for 20 min in a buffer containing 25 mM Tris/maleate, pH 7.0, 0.6% bovine serum albumim, 1 mM dithiothreitol and 1 µM p(NH)ppG.

Stage 2. The incubating mixture was divided into two parts: one was irradiated for 10 min and the other was kept in a dark compartment at 4°C (control system).

Stage 3. The two suspensions (irradiated and control) were acidified by adding (0.5 vol.) 0.2 M sodium acetate (final pH 4.0), and kept at 4°C for 10 min.

Stage 4. The incubation systems were neutralized by adding 1 M Tris/base (1/16 vol.). The pH change was verified by the color change of phenol red included in the reaction mixture.

Stage 5. The contents of each reaction vessel was divided into two portions: one added to a test tube containing adenylate cyclase assay reagents and ^{125}I-labeled hCG for simultaneous determination of hCG binding and adenylate cyclase activity. The second portion was added to a test tube containing the same reagents but in addition also an excess of 800 nM unlabeled hCG, used to determine the nonspecific binding of ^{125}I-labeled hCG. All four systems were incubated at 30°C and samples were withdrawn at the indicated times to determine ^{125}I-labeled hCG binding, nonspecific ^{125}I-labeled hCG binding, and adenylate cyclase activity. Samples for these determinations were also taken before initiation of step 1 and subsequent to each of the stages listed.

Results

Inactivation of hCG receptor by high-intensity light

Purified ovarian plasma membranes contain sites which specifically bind hCG. Exposure of these plasma membrane suspensions to high-intensity light at 4°C resulted in a gradual decrease in their binding capacity (Fig. 1 and Table I). 50% of the initial binding capacity (1.26 pmol/mg plasma membrane

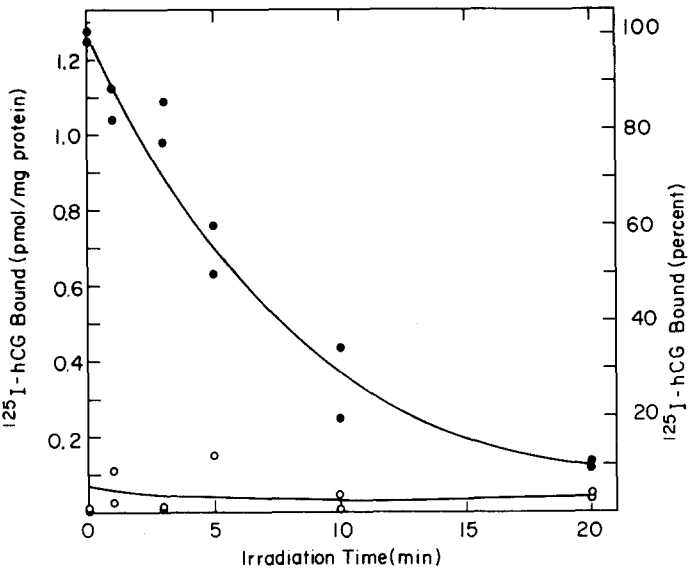

Fig. 1. Photoinduced inactivation of the hCG binding site. Purified ovarian plasma membranes were exposed to light irradiation for various times. The residual ^{125}I-labeled hCG (^{125}I-hCG) binding activity was assayed with 2.4 nM ^{125}I-labeled hCG in the absence (•) or presence (○) of excess (400 nM) unlabeled hCG.

TABLE I

SUMMARY OF EFFECTS OF HIGH-INTENSITY LIGHT IRRADIATION ON THE ADENYLATE CYCLASE SYSTEM IN RAT OVARIAN PLASMA MEMBRANES

Values characteristic to damage induced in ovarian adenylate cyclase by high-intensity light are given. Differential susceptibility to light irradiation within the enzyme system is characterized by different half-lives (t_{50};min) that indicate the time of exposure that resulted in 50% loss in activity. The enzyme was assayed following irradiation in the absence (basal) or presence of NaF, p(NH)ppG or hCG as described in Fig. 4. The amount of activity that remained after 20 min of exposure was also taken as an indicator for stability of the respective components. The same parameters were used to evaluate the stability of the binding sites for hCG and p(NH)ppG. Due to high stability of the preformed complexes with hCG and p(NH)ppG the table lists only the amount remaining after 20 min of irradiation. Adenylate cyclase activity of a preformed complex with hCG is also given. Values are mean ± S.E. and numbers of experiments are in parentheses. n.d., not determined.

Activity		Half-life (t_{50}; min)	Residual activity following 20 min of exposure (% of non-irradiated control)	
			Plasma membranes	Preformed complex
Adenylate cyclase	Basal	5.9 ± 1.2 (5)	22 ± 4.9 (5)	n.d.
	NaF	18 ± 1.2 (4)	39 ± 5.7 (4)	n.d.
	p(NH)ppG	17; > 20	n.d.	n.d.
	hCG	5.9 ± 0.6 (7)	4.7 ± 1.2 (7)	3.6 ± 1 (4)
^{125}I-labeled hCG binding		5.1 ± 0.8 (3)	18.5 ± 5.3 (4)	77.7 ± 4.3 (10)
[8-^{3}H]p(NH)ppG binding		13.5 ; 10	44.9; 44.4	97 ± 2.7 (3)

protein) was destroyed within 6 min and only 10% of the specific sites remained after 20 min of irradiation (see also Table I). Control systems assayed in the presence of excess unlabeled hCG were not affected by light irradiation.

Photo induced inactivation of hCG receptors: protection by hCG

A plasma membrane suspension was preincubated under standard conditions with 0.5 nM ^{125}I-labeled hCG for 20 min at 30°C to form the receptor-hCG complex. The amount of complex formed was determined on a sample and the rest of the suspension was equilibrated to 4°C. A control suspension was incubated in the absence of ^{125}I-labeled hCG. Subsequently suspensions were exposed to light and samples were withdrawn at various times and transferred to a dark compartment at 4°C. The amount of ^{125}I-labeled hCG-receptor complex that remained, as compared to the residual binding activity of the control membrane was determined (Fig. 2). Only 11% of the bound hormone were released following 5 min irradiation in the presence of 0.5 nM hCG, whereas over 70% of the receptor sites were inactivated within 5 min in a control suspension which was irradiated in the absence of hCG. Following 20 min of exposure one could still recover close to 80% of the protected complex but over 85% of the unoccupied receptors were lost (see also Table I).

The effect of high-intensity light irradiation on the response of rat ovarian adenylate cyclase to stimulation by hCG

Hormone-sensitive adenylate cyclase in rat ovarian plasma membranes is

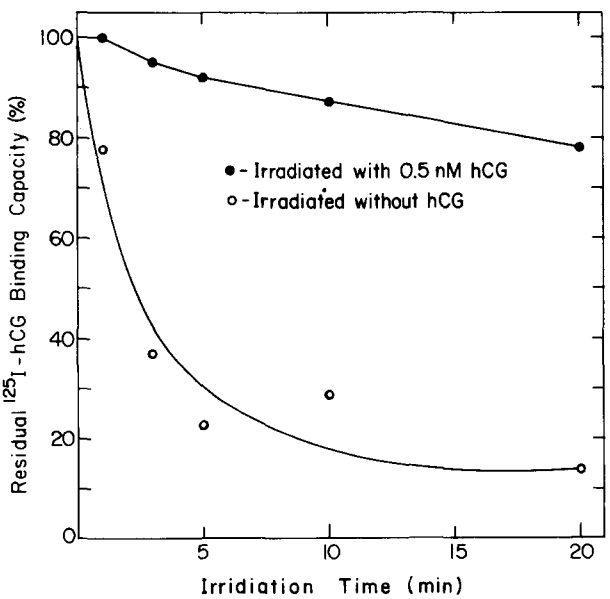

Fig. 2. Photoinduced inactivation of the hCG binding site: Protection by hCG. Plasma membrane suspensions were preincubated with 0.5 nM ^{125}I-labeled hCG (^{125}I-hCG) in a final volume of 0.8 ml. The suspensions were exposed to irradiation and samples (50 µl) were withdrawn at various times and filtered. The amount of ^{125}I-labeled hCG that remained bound following preincubation with 0.5 nM ^{125}I-labeled hCG (●) is shown. In addition, plasma membrane suspension was irradiated in the absence of ^{125}I-labeled hCG and residual binding activity (○) after exposure for various times was assayed in the presence of 2.5 nM ^{125}I-labeled hCG.

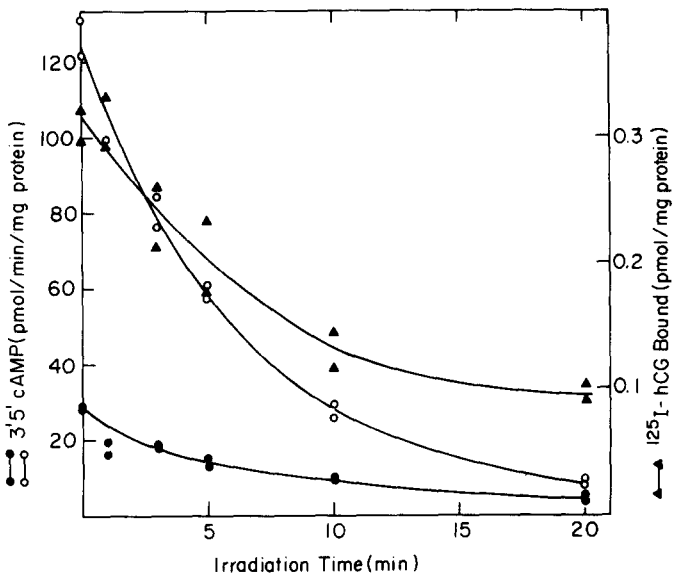

Fig. 3. Inactivation of the hCG-sensitive adenylate cyclase by irradiation. Plasma membranes were suspended in a medium containing 25 mM Tris/maleate pH 7.0 and 0.6% bovine serum albumim and were irradiated for various times. The residual adenylate cyclase activity was determined in the presence of 10 μM GTP (●) or 1.4 nM hCG plus 10 μM GTP (○). The residual binding activity was determined in the presence of 1.4 nM ^{125}I-labeled hCG (^{125}I-hCG) (▲).

stimulated over 4-fold by 1.4 nM hCG (Fig. 3.) Irradiation of a plasma membrane suspension with light under conditions specified in Materials and Methods induced a decline in hCG-stimulated activity (pmol cyclic AMP/min per mg protein) from 125 to 62 within 5 min. Following 20 min of exposure hormone-dependent activity was almost totally abolished (greater than 92%). Hormone-independent enzyme activity was somewhat more resistant declining from 29 to 17 pmol cyclic AMP/min per mg protein within 5 min of irradiation. Activity ratio decreased from 4 (prior to irraiation) to 2 following 20 min of exposure. To compare the decay in responsiveness of adenylate cyclase to hCG with the decay in the hCG binding activity, we determined simultaneously residual binding of ^{125}I-labeled hCG to the same plasma membrane suspension (Fig. 3) It was found that high-intensity light decreased ^{125}I-labeled hCG binding with a similar time course.

We then investigated whether any of the known ligands of this enzyme system can protect adenylate cyclase activity against the radiation damage. Plasma membrane suspensions were exposed to light in the presence of several assay constituents at concentrations normally present in the assay system, i.e. ATP, Magnesium acetate, Tris/acetate and dithiothreitol; NaF; cyclic AMP; hCG with or without p(NH)ppG alone. None of these additions had any protective effect when hormone-dependent or independent adenylate cyclase activity was assayed. It should be pointed out that at the same time hCG efficiently protected the binding capacity of the hCG receptor as previously shown (Fig. 2). This protective effect of hCG was also seen in the presence of p(NH)ppG (Fig. 5). Exposure to light of hCG alone in solution had no significant effect on

subsequent activity of the hormone. Over 80% of the activity, as judged by adenylate cyclase activation, could be recovered following 20 min of exposure and hormone was therefore used in large excess whenever possible.

The response of ovarian adenylate cyclase to various stimulants following high-intensity light irradiation

A plasma membrane suspension was irradiated, under standard conditions. Samples were withdrawn at various times and transferred to a dark compartment kept at 4°C. Residual adenylate cyclase activity was then determined in the presence of several hormones naturally involved in ovarian function, i.e. LH, hCG, FSH or prostaglandin E_2, as well as in the presence of NaF or p(NH) ppG (Fig. 4).

It was shown that hormone-independent activity, stimulated by NaF or p(NH)ppG, was almost completely resistant to irradiation for the first 10 min (Fig. 4, Table I) but thereafter declined somewhat. 60% of NaF-stimulated activity and 75% of p(NH)ppG-stimulated activity remained following 20 min of irradiation. In contrast, adenylate cyclase activity due to stimulation by LH, hCG, FSH or prostaglandin E_2 decayed rapidly, with more than 90% inactivation observed at 20 min of irradiation. This finding is in agreement with the results obtained for hCG (Fig 3). The rate of decay differed for the various

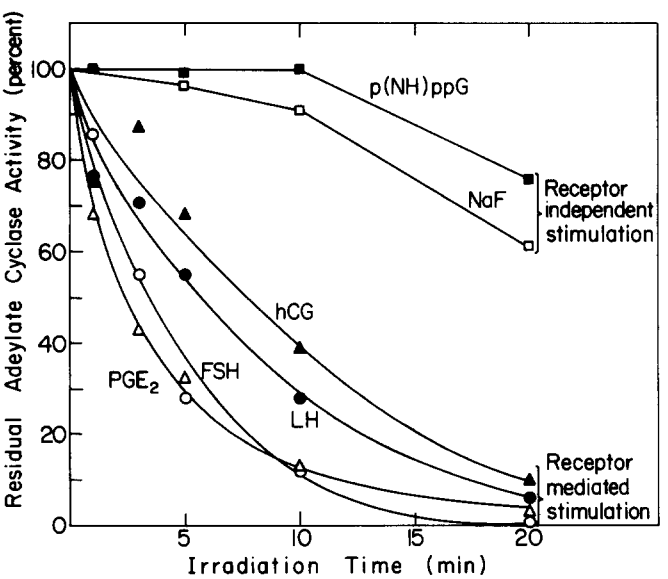

Fig. 4. Rat ovarian adenylate cyclase activity in the presence of various stimulants following light irradiation. A plasma membrane suspension was irradiated for various times and adenylate cyclase activity was subsequently assayed. The absolute activity (pmol cyclic AMP/min per mg protein) of control untreated membranes in the presence of the following additions was: ●, 10 nM LH, 212; ○, 10 nM FSH, 145; ▲, 10 nM HCG, 157; △, 40 μM prostaglandin E_2 (PGE_2), 121; ■, 10 μM p(NH)ppG, 70 and □, in the presence of 10 mM NaF, 146. Stimulation of the adenylate cyclase by the hormones was determined in the presence of 10 μM GTP, which on its own generated an activity of 37 pmol/min per mg protein. Activity of untreated enzyme was regarded as 100%. The change in adenylate cyclase activity in the presence of GTP alone was similar to that described in Fig. 3.

hormone-sensitive activities, with an apparent decreasing order of stability, prostaglandin E_2 < FSH < LH < hCG (two observations).

Binding of hCG and stimulation of adenylate cyclase in plasma membranes irradiated in the presence of hCG

Binding of hCG to its receptor does normally not involve covalent binding between these molecules. However, exposure to light may have induced the formation of intermolecular covalent bonds between proteins and thus explain the high stability of the hCG-receptor complex to light. In order to test this possibility, we irradiated membrane particles containing bound ^{125}I-labeled hCG for 10 min (Fig. 5A) and subsequently incubated them at 4°C (pH 4.0)

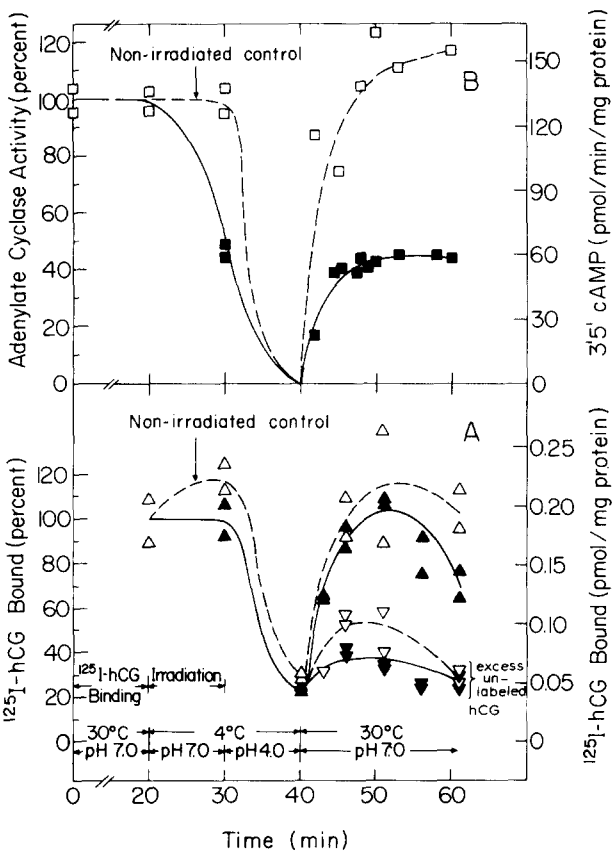

Fig. 5. Protection of the hCG receptor from irradiation damage, its ability to rebind ^{125}I-labeled hCG (A) and the failure of the new formed complex to stimulate adenylate cyclase (B). A plasma membrane suspension was preincubated with 1.4 nM ^{125}I-labeled hCG and 10 mM NaF for 20 min at 30°C. The suspension was divided into two portions: one was irradiated for 10 min and the other was kept in the dark (control). The bound hormone was dissociated following acidification. Binding of hCG (A) in the control neutralized suspensions (△, ▽, □) and irradiated suspensions (▲, ▼, ■) was determined with 1.4 nM ^{125}I-labeled hCG in the absence (△, ▲) or in the presence of 800 nM unlabeled hCG (▽, ▼). The amount of plasma membrane protein per assay was 10 μg. Adenylate cyclase activity (B) in the presence of 10 μM p(NH) ppG, 10 mM NaF, and 1.4 nM hCG was determined in the irradiated suspension (■) and in the control suspension (□). Plasma membrane protein per assay was 3.1 μg. Activity of adenylate cyclase before irradiation was taken as 100%.

for 10 min. Under these conditions, nearly 75% of the bound ^{125}I-labeled hCG dissociated from irradiated and control non-irradiated membranes. This situation was fully reversed upon neutralization and transfer of the reaction mixture to 30°C. Reuptake of ^{125}I-labeled hCG was prevented by the addition of an excess (600-fold) of unlabeled hCG. Similar results were obtained with control membranes which were not exposed to high-intensity light irradiation. Along with restoration of the original level of hCG-receptor complex (following neutralization) the activity of hormone-sensitive adenylate cyclase was also restored but only in control membranes (Fig. 5B). No renewed hormone-stimulated adenylate cyclase activity could be observed in hCG-protected light-treated membranes (see also Table I). However, as shown in Fig. 5B, nearly 50% of the original adenylate cyclase activity could still be observed prior to acidification, provided 10 mM NaF was also present in addition to 1.4 nM hCG. The same activity level remained following neutralization.

Sensitivity of the guanosine nucleotide binding site to irradiation

Ovarian plasma membranes contain binding sites specific for guanosine nucleotides. In the experiment shown in Fig. 6, plasma membranes were irradiated for various periods of time and residual nucleotide binding activity was determined. Untreated plasma membranes bound 26 pmol of [8-^{3}H]p(NH)ppG/mg membrane protein. Upon irradiation, a gradual decrease in the number of binding sites was observed reaching a final value of 11 pmol/mg protein after 20 min of exposure. In other experiments (Table I) the nucleotide was bound to plasma membranes prior to exposure to high-intensity light irradiation. Under these conditions, no label was released in response to light irradiation

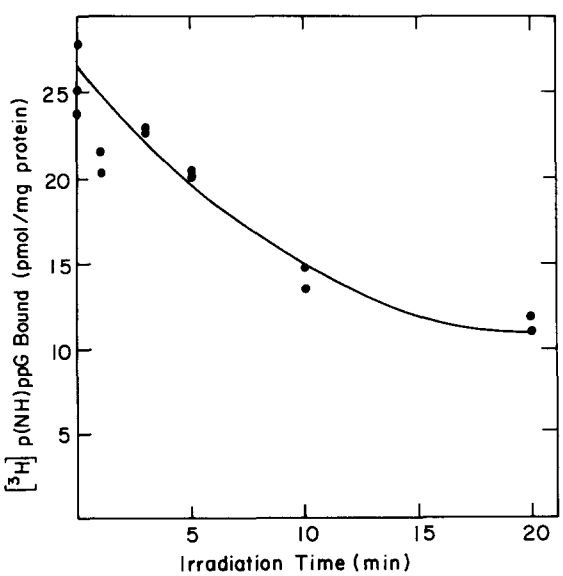

Fig. 6. Photoinduced inactivation of the p(NH)ppG binding site in the ovarian plasma membrane. A plasma membrane suspension was irradiated for the times indicated on the abscissa. Samples were removed and residual [8-^{3}H]p(NH)ppG binding was determined. The concentration of [8-^{3}H]p(NH)ppG used was 0.18 μM.

(20 min). Similar results were obtained using [8-^{3}H]p(NH)ppG, [8-^{3}H]GTP or [α-^{32}p]GTP. However treatment of the plasma membrane suspension with 5% trichloroacetic acid after irradiation released all the bound [α^{32}-p]GTP.

Discussion

In these studies, we showed that high-intensity light effectively reduced the response of ovarian adenylate cyclase to LH, FSH and prostaglandin E_2. This effect was selective, in that catalysis, binding of regulatory ligands and the interaction between components of the enzyme system were affected at different rates. We defined the following events in the stimulation of adenylate cyclase and related them to activity of individual components of the enzyme system.

Basal activity is related to intrinsic expression of the catalytic component. Stimulation by NaF or p(NH)ppG involves the mediation of the guanosine nucleotide regulatory component (GF) [3]. Stimulation by the hormone is a receptor-mediated event whereby hormone binding is coupled to adenylate cyclase activation. Binding of ^{125}I-labeled hCG and [8-^{3}H]p(NH)ppG are independent measures of the interaction of these ligands with the hormone receptor and guanosine nucleotide binding protein, respectively.

Hormone binding and stimulation of adenylate cyclase were affected by exposure to light in a similar time course (Fig. 3), suggesting that reduced response of the enzyme may result, at least in part from destruction of receptors. The higher resistance of hormone-independent activity (Table I, Figs. 3 and 4) is consistent with this finding.

Constituents of the adenylate cyclase assay (i.e. nucleotides, metal ions hormone, etc.) did not stabilize the enzyme or its response to hormones. By contrast, hCG effectively prevented the light-induced damage to the binding site, (Table I, Fig. 2) and the protected receptor retained the ability to rebind hormone to the same extent observed with non-irradiated control membranes (Fig. 5). However, the ability of the receptor-hormone complex to stimulate adenylate cyclase was not conserved (Fig. 5, Table I). These observations therefore permit a distinction between two functions of the hormone receptor: (i) hormone binding, and (ii) interaction of the occupied hormone receptor with other membrane component(s) that leads to activation of adenylate cyclase. Protection of the hCG receptor seems to be specific for hCG since FSH at saturating concentration (10 nM) did not prevent the radiation damage. It is likely that the protective effect observed will also be seen with LH preparations of various sources. The determination of such an effect may be used as an independent measure for the interaction of these hormones with the membrane receptor and for analysis of consequent conformational changes induced in the receptor. Furthermore, studies of this kind may contribute to better understanding of the structure and function relationship of these hormone molecules.

There may be at least two distinct sites in the adenylate cyclase system which are affected by high-intensity light irradiation. One is associated with the receptor itself and accounts for the loss of hormone binding (Fig. 1, Table I) and hormonal stimulation of the enzyme (Figs. 3 and 4). This site seems to be

protected from radiation damage when the receptor is occupied by hormone, as judged by retention of bound ^{125}I-labeled hCG (Fig. 2) and conservation of the ability to specifically reassociate with ^{125}I-labeled hCG following exposure to high-intensity light (Fig. 5). Under these conditions a second site of damage becomes apparent. At this stage hormone binding does not lead to stimulation of adenylate cyclase even in the presence of p(NH)ppG (Table I). In contrast, stimulation by NaF is still possible and to the same extent, before acidification and after neutralization (Fig. 5). This suggests that the GF-type regulatory subunit [3] believed to be tightly associated with the enzyme [6] functions properly. The selective regulation by GF as seen here may be attributed to the fact that p(NH)ppG association with the GF subunit is hormone dependent to a large extent whereas NaF stimulation is not. This locates the second site of damage distal to hormone binding but proximal to GF subunit stimulation. This site may well be still associated with the receptor molecule. Another reasonable candidate to mediate receptor regulation of the GF subunit is a receptor-associated guanosine nucleotide site proposed for the glucagon-sensitive adenylate cyclase by Welton et al. [26] and by Lad et al. [8]. These authors demonstrated the existence of two functionally distinct guanosine nucleotide sites. These are believed to be associated with the hormone receptor and adenylate cyclase, respectively. Inactivation of such a receptor-associated intermediary component or reducing its ability to interact with the receptor are therefore compatible with our findings. However, the existence of two forms of guanosine regulatory sites in glycoprotein hormone-sensitive adenylate cyclase as also suggested [27] has yet to be demonstrated.

The stability to irradiation of the bulk of GTP binding sites (Fig. 6) is also reflected in the relative resistance of the stimulatory effect of p(NH)ppG on adenylate cyclase and may include the GF component only. One-half of the p(NH)ppG binding activity remained after 20 min of exposure to light (Fig. 6) and over 97% [8-^{3}]p(NH)ppG remained bound if the nucleotide-plasma membrane complex was irradiated for 20 min (Table I).

High-intensity light irradiation ($\lambda > 300$ nm) may induce covalent cross-linking between proteins, between proteins and nucleotides, or may modify certain amino acid residues. Sperling and Havron [13,28] presented evidence that exposure of pancreatic ribonuclease to high-intensity light ($\lambda > 300$ nm) in the presence of certain pyrimidine nucleotides induces covalent cross-linking of these nucleotides to the enzyme and consequently resulting in the inactivation of the enzyme. Likewise incubation of ATP with histone H4 resulted in the incorporation of labeled nucleotide into a specific peptide of the protein molecule [18]. The interaction of the nucleotides with the protein molecules was thought to occur at or close to the respective binding site.

Light-induced cross-linking of endogenous nucleotides to components of adenylate cyclase upon irradiation is a possible mechanism for the observations presented here. However, incubation of plasma membranes with labeled [8-^{3}H] p(NH)ppG (Fig. 6) and [α-^{32}p]GTP or [8-^{3}H]GTP resulted in their specific binding but no significant amount of the bound nucleotides was released upon subsequent exposure to light. Moreover, treatment with trichloroacetic acid released all of the bound radioactivity indicating that no massive incorporation of added nucleotides to membrane components has occured. It cannot be

excluded, however, that endogenous nucleotides present in these membrane preparations and that do not readily exchange with exogenous labeled nucleotides are responsible for the observed effects. [α-^{32}p]GTP becomes cross-linked to purified rat brain tubulin under these conditions indicating that GTP, like ATP [18], may become covalently linked to proteins as a result of light irradiation (Azulai, R. and Salomon Y. unpublished results). These preliminary experiments also revealed that tubulin is partly degraded as a result of light irradiation, suggesting that degradation of membrane proteins could also explain some of the observations decribed here.

Radiation damage is not restricted to the LH/hCG receptor (Fig. 4). Stimulation of adenylate cyclase in these membranes by FSH and prostaglandin E_2 is also sensitive to light. Experiments are underway to determine wheter common sites of damage are responsible for loss of responsiveness to all of these hormones. Spectral analysis of these phenomena may lead to identification of the reactive groups affected and will advance our understanding of how hormone receptors regulate adenylate cyclase.

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