Selective Photoinduced Uncoupling of the Response of Adenylate Cyclase to Gonadotropins by 5-Iodonaphthyl 1-Azide†

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ABSTRACT: 5-Iodonaphthyl 1-azide (INA) has been previously shown to selectively label, on photolysis, only those proteins in contact with the membrane lipids. Low concentrations (<10 μM) of INA added to rat ovarian plasma membranes induced, on photoactivation, a selective and complete loss of the response of the adenylate cyclase to stimulation by human chorionic gonadotropin (hCG) or luteinizing hormone (LH). In contrast, this treatment affected neither hCG binding to the receptor nor the stimulation of the enzyme by NaF. That the uncoupling of the receptor from the enzyme by INA occurred within the lipid bilayer can be derived from the finding that the prior presence neither of saturating concentrations of hCG nor of the aqueous nitrene-scavenger glutathione (GSH) prevented this effect. Photolysis at higher concentrations of INA (0.1–1 mM) led to the inhibition of the adenylate cyclase stimulated by fluoride. This effect was totally prevented by glutathione. A similar behavior was obtained with a water-soluble analogue of INA, namely, 5-diazonionaphthyl 1-azide (DAN). On photoactivation with 30 μM DAN, the NaF-stimulated adenylate cyclase was inhibited, but this effect was completely prevented by added GSH. At low concentrations where its effects are restricted to the lipid core, INA may represent a useful tool to define receptor coupling with the adenylate cyclase. The capacity of INA at low concentrations to uncouple the hormone receptor from the adenylate cyclase is not restricted to the LH/hCG receptor. Other hormone receptors tested behaved similarly. Therefore, the reported findings appear to represent a general phenomenon.

The selective labeling of the lipid-associated domains of membrane proteins by means of photoactivatable lipophilic compounds has been demonstrated (Klip & Gitler, 1974; Bayley & Knowles, 1978a,b). 5-Iodonaphthyl 1-azide (INA)[1] was shown to be effective by virtue of its distinctive properties (Bercovici & Gitler, 1978; Gitler & Bercovici, 1980; Sidman et al., 1980; Bayley & Knowles 1980): (i) It has a high partition coefficient (>10^5) into the liquid lipid phase of biological membranes. (ii) Upon photoactivation, the generated nitrene is confined only to the lipid phase of the membrane. Thus, the extent and pattern of labeling of all membranes studied were not affected by the presence, during photolysis of high concentrations, of a water-soluble nitrene scavenger such as glutathione. (iii) At low concentrations, INA incorporates mainly and nonreversibly into proteins.

Adenylate cyclase is an enzyme that responds to hormonal stimuli transduced through cell membranes, with the hormone receptor on the outer surface and the regulatory and catalytic subunits facing the cytoplasmic side of the membrane. The present knowledge regarding the structure and function of this complex enzyme system, as well as the regulatory aspects of its components, has been reviewed in recent years (Limbird, 1981; Cooper, 1982; Dumont et al., 1980). In this study, we have examined how a lipid-soluble reactive reagent such as INA can affect the adenylate cyclase system. We have systematically studied the effect of various concentrations of INA and an equivalent water-soluble azide, 5-diazonionaphthyl 1-azide (DAN), on the adenylate cyclase system. Our results indicated that INA at low concentrations is confined to the lipid core and thus on photoactivation can selectively uncouple the receptor from the catalytic subunit. At higher concentrations, the reagent becomes less selective and affects components present at the membrane–water interface. This can be derived from the fact that a water-soluble analogue of INA, DAN, mimics the effects of high INA concentrations in that it inhibits the NaF-stimulated adenylate cyclase, but its effect is prevented completely by glutathione.

Materials and Methods

Reagents, enzymes, nucleotides, radiolabeled [^{3}H]-3′,5′-cAMP, [α-^{32}P]ATP, carrier free ^{125}I, ovine luteinizing hormone (LH), and human chorionic gonadotropin (hCG) were as described previously by Ezra & Salomon (1980). Guanosine 5′-*O*-(3-thiotriphosphate) was obtained from Boehringer, Mannheim.

Synthesis of INA. INA was synthesized from 5-aminonaphthyl azide as described previously (Bercovici & Gitler, 1978). INA was solubilized in ethanol, and the concentration was determined spectrophotometrically at 310 nm with the assumption of a molar extinction coefficient (ε) of 20 000 M^{-1} (Klip & Gitler, 1974; Bayley & Knowles, 1978a,b).

The solution was stored at −20 °C in the dark. For preparation of [^{125}I]INA, carrier-free Na^{125}I was used, and the final product was used within 7 days.

Treatment of Membranes with INA. Purified plasma membranes were suspended in ice-cold 10 mM Tris–acetate buffer, pH 7.5 (buffer A), to final concentration of 0.2 mg of protein/mL. Concentrated INA solution (5 mM in ethanol) was added to the membrane suspension to the desired final concentration. The final proportion of ethanol did not exceed 2% v/v in the membrane suspension. The reaction mixture was then kept on ice for 5 min. Unless otherwise indicated, membrane suspensions were exposed to light for 90 s in a "Wild" type projector equipped with a 200-W HBO lamp. The distance between the light source and the membrane suspension was 10 cm. The light was filtered to allow transmittance in the wavelength range 300 nm < λ < 400 nm, with Corning

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[1] Abbreviations: 3′,5′-cAMP, adenosine cyclic 3′,5′-phosphate; BSA, bovine serum albumin; DAN, 5-diazonionaphthyl 1-azide; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FSH, follicle-stimulating hormone; G/F, guanine nucleotide binding protein; GSH, glutathione; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); hCG, human chorionic gonadotropin; INA, 5-iodonaphthyl 1-azide; oLH, ovine luteinizing hormone; Tris, tris(hydroxymethyl)aminomethane.

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filters, types 0-54 and 7-54 in combination. In some experiments, light irradiation was carried out in the presence of 15 mM reduced glutathione (GSH). A stock solution of 150 mM GSH was neutralized to pH 7.5 with $NaHCO_3$ and was added to the membrane suspension prior to photolysis.

After irradiation, membranes were assayed for adenylate cyclase activity and/or binding of [^{125}I]hCG. In each experiment, two types of controls were used. (1) Control with no INA: Ethanol was added to the membrane suspension to a final concentration of 2% v/v and the membranes were irradiated. (2) Dark control: Membranes were treated with INa as described above but were left in dark (not irradiated).

Synthesis of DAN. DAN was obtained as described elsewhere (Bercovici & Gitler, 1978) and was solubilized in 2 N H_2SO_4, and its concentration was determined spectrophotometrically at 345 nm with a molar extinction coefficient of 20000 M^{-1}. The solution was stored at −20 °C. Shortly before use, DAN solution was titrated to pH 7.5 with $NaHCO_3$, and its concentration was adjusted with water to 3 mM.

Treatment of Membranes with DAN. Membranes were suspended in 10 mM Tris–acetate buffer, pH 7.5, as described for INA treatment. A 3 mM DAN solution, pH 7.5, was then added to the membrane suspension to the desired final concentration.

Exposure to light was performed as described for INA treatment. Controls were designed as for INA treatment. Control with no DAN contained a proper dilution of neutralized H_2SO_4 (Na_2SO_4).

Adenylate Cyclase Assay. Adenylate cyclase activity was determined by measuring [^{32}P]-3′,5′-cAMP formation from [α-^{32}P]ATP. Incubation conditions and isolation of 3′,5′-cAMP were as described previously (Salomon, 1979), except that the incubation time was 10 min. All assays were performed in triplicate, and mean ± SEM is given. Basal enzyme activity was determined in the presence of 10 μM GTP. When added, the concentration of NaF was 10 mM, that of oLH was 0.1 μM, and that of hCG was 20 nM. Plasma membrane concentration was 4 μg of protein/assay. All assays were carried out in a darkroom illuminated by red light. One unit of enzyme is defined as the amount of protein catalyzing the formation of 1.0 pmol of 3′,5′-cAMP/min at 30 °C.

Labeling of hCG with ^{125}I. Highly purified hCG was used as the substrate for iodination. Iodination was performed with lactoperoxidase according to the method described by Miyachi et al. (1972). The labeled [^{125}I]hCG was isolated by gel filtration on a Sephadex G-25 (medium) column. Specific activity of [^{125}I]hCG was established by the ability of the iodinated hormone to activate adenylate cyclase in comparison with a calibration curve prepared with native hCG. Specific radioactivity obtained was 12–24 mCi/mg.

Binding of [^{125}I]hCG to Plasma Membranes. Binding of [^{125}I]hCG to ovarian plasma membranes was measured by Millipore filtration essentially as described previously (Amir-Zaltzman & Salomon, 1980) except that bovine serum albumin (BSA) was omitted from the incubation mixture because it interacts with INA (Bercovici et al., 1978). Incubation in a final volume of 50 μL contained 25 mM Tris–acetate (pH 7.5) and [^{125}I]hCG [10 nM, (0.5–1.5) × 10^5 cpm]. The binding was initiated by the addition of ovarian plasma membranes (10 μg of protein). The reaction was terminated after 20-min incubation at 30 °C with the addition of 5 mL of 2.5% BSA in 25 mM Tris–acetate, pH 7.5. The samples were filtered through Millipore filters (0.45-μm–25-mm diameter, presoaked overnight in 5% BSA solution) and washed once with 5 mL of a solution containing 1% BSA in the same buffer. Washing was repeated twice with 2 mL of the same solution, and the radioactivity retained on the filters was counted. Nonspecific binding was determined in the presence of an excess (800 nM) of nonlabeled hCG. The extent of nonspecific binding was found to be 0.5–1% of the total radioactivity introduced to the assay. The binding to the membranes was 10–12% of total radioactivity introduced.

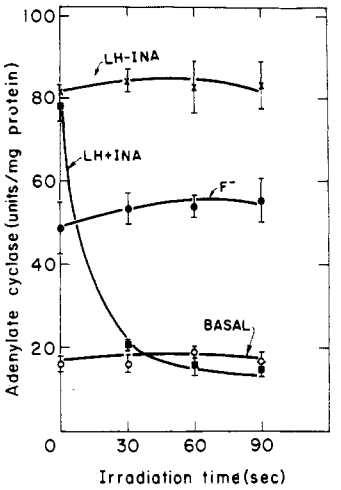

FIGURE 1: Influence of irradiation on plasma membranes. Membranes were treated with INA as described under Materials and Methods. Final concentration of INA was 10 μM (30 nmol/mg of membrane protein). Following exposure to light for the indicated time period, adenylate cyclase activity was determined in the dark. Basal and F^--stimulatory activity were measured in the presence of 10 μM INA. Activity in response to LH was determined in the presence (LH + INA) or absence (LH − INA) of INA. The value obtained for the dark control in this experiment is indicated at time = 0.

Plasma Membrane Preparation. Preparation of purified plasma membranes from ovaries of immature rats primed with pregnant mare serum gonadotropin was performed as described previously by Mintz et al. (1978).

Results

Determination of Dose of Light Required for Photoactivation of INA. The procedure used in this study for evaluation of the effects of INA on LH/hCG-sensitive adenylate cyclase consists of three steps: (i) incubation of plasma membranes with INA in the dark, (ii) exposure to light for photoactivation of INA, and (iii) assay of residual enzyme activity. In the experiment described in Figure 1, we determined the dose of light required for photoactivation of INA (step 2) following incubation of the membranes with 10 μM INA for 5 min. Activity of adenylate cyclase was determined in the absence (basal) or presence of fluoride ions or LH. It can be seen that treatment of plasma membranes with INA at this concentration induces a selective loss of the response of adenylate cyclase to LH. This effect is completed following 30 s of light irradiation (LH + INA). Treatment of the membranes with BSA solutions (Bercovici et al., 1978), which removes quantitatively the unbound INA photolysis products, does not affect the observed inhibition (data not shown).

In contrast, the response of the enzyme to LH is not affected by light irradiation in the absence of INA (LH − INA). Likewise, basal activity, the response of the enzyme to F^- ions and to guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (not shown), was unaffected by treatment with INA in the dark or following exposure to light. All further studies described were conducted at an exposure time of 90 s.

Effect of Low Concentrations of INA on Receptor Cyclase Coupling. The activity of the adenylate cyclase was studied following incubation of the membranes with increasing con-

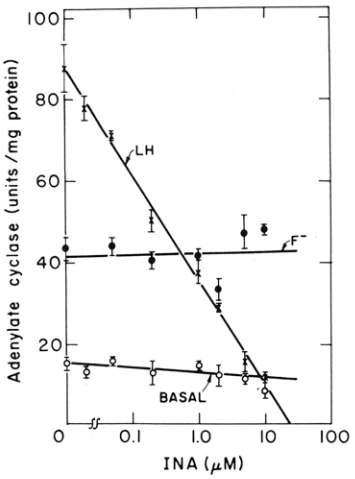

FIGURE 2: Influence of increasing concentration of INA on adenylate cyclase activity. Membranes were treated with increasing concentrations of INA as described under Materials and Methods. Irradiation time was 90 s, residual basal activity and activity in response to NaF and LH were then determined. Control with no INA is represented as activity at 0 INA concentration.

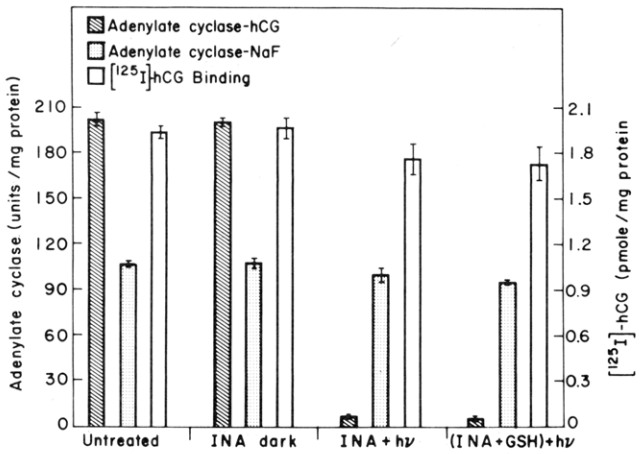

FIGURE 3: Effect of INA on adenylate cyclase and [^{125}I]hCG binding in the presence and absence of reduced glutathione. Membranes were incubated with 16.5 μM INA (45 nmol/mg of protein). Subsequently, membranes were divided into three test tubes, 450 μL each. One (dark control) received 50 μL of buffer A and was not exposed to light. The second (INA + $h\nu$) received 50 μL of buffer A and was exposed to light. To the third [(INA + GSH) + $h\nu$] was added 50 μL of GSH (150 mM) in buffer, and this suspension was irradiated as described under Materials and Methods. Final concentration of INA in each tube was 15 μM. Final concentration of GSH in the third tube was 15 mM. Residual adenylate cyclase activity and hCG binding were done as described under Materials and Methods.

centration of INA. Membranes were first incubated with 0.02–10 μM INA and subsequently irradiated for 90 s (Figure 2). In agreement with the results seen in Figure 1, the effect of INA was selective, i.e., abolishing only the response of the enzyme to LH. The half-maximal effect was seen at 1 μM INA (3 nmol/mg of membrane protein). No change was observed at these concentrations in the response of the enzyme to NaF or in the level of basal activity.

Localization of Site Modified by INA. Since photolysis of INA affected only the response of adenylate cyclase to the hormone, it was reasonable to assume that INA may affect the binding of the hormone to the membrane receptor. We therefore tested [^{125}I]hCG binding and the response of adenylate cyclase to hCG in membranes treated with 10 μM INA (Figure 3). It was found that while INA totally abolished the response of adenylate cyclase to hCG, binding of the hormone to the membrane receptor was unimpaired.

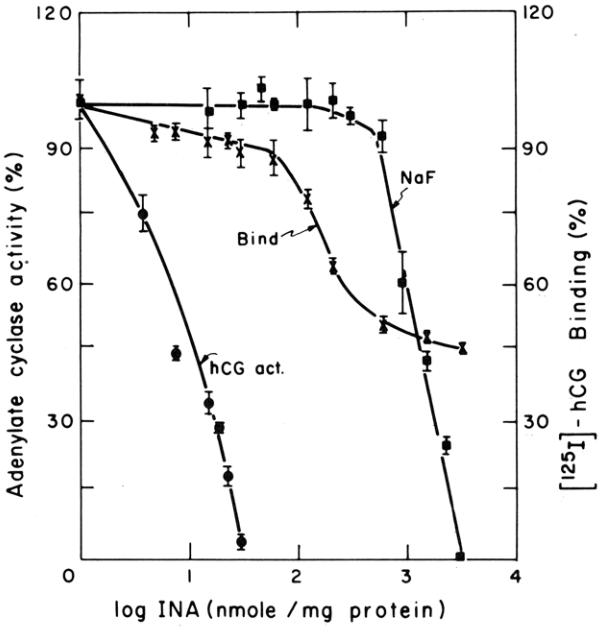

FIGURE 4: Effect of high concentration of INA on adenylate cyclase system. Membranes were treated with increasing concentrations of INA as indicated. Activity of adenylate cyclase in response to hCG or NaF and [^{125}I]hCG binding were measured as described under Materials and Methods. 100% was taken as activity in membranes not treated with INA (0 INA concentration).

The nitrene derivative generated by photolysis of the azide moiety of INA can be scavenged by interaction with free thiols. The use of hydrophilic thiols such as glutathione (GSH) will therefore permit the distinction between nitrenes generated within or outside the lipid bilayer of the membrane (Bayley & Knowles, 1978a; Jørgensen et al., 1982). The effect of 10 μM INA was therefore tested on adenylate cyclase activity and on [^{125}I]hCG binding in the presence of 15 mM GSH (Figure 3). It was found that GSH did not interfere with the action of INA at this concentration. Thus, INA still abolished the response of adenylate cyclase to the hormone in the presence of 15 mM GSH.

In another experiment (not shown), we found that prebinding of hCG to the membrane prior to INA treatment did not protect the responsiveness of adenylate cyclase to stimulation by this hormone following treatment with INA. The findings in these experiments indicate (1) that the site of INA action is located within the lipid bilayer of the membrane and (2) that INA does not affect the system by preventing hormone receptor complex formation.

Effects of High Concentrations of INA on Adenylate Cyclase System. The experiments presented thus far support the view that at low concentrations (<10 μM), the action of INA takes place solely in the hydrophobic region of the membrane. It seemed likely that increasing the concentrations of INA (>10 μM), could affect additional sites. In an attempt to identify such sites, we treated membranes with INA concentrations markedly higher than those used thus far (cf. Figure 2). Loss in hormone responsiveness (as already seen in Figures 1 and 2) occurs at low concentrations and was completely abolished at an INA concentration of 30 nmol/mg of protein (Figure 4). Further increase of INA concentrations did not affect the response of the enzyme to NaF even at concentrations as high as 1000 nmol/mg of protein. However, higher INA concentration resulted in a gradual loss in responsiveness to NaF and completely abolished the activity of adenylate cyclase at 3000 nmol/mg of protein. The binding of [^{125}I]hCG was only slightly affected (10%) by INA con-

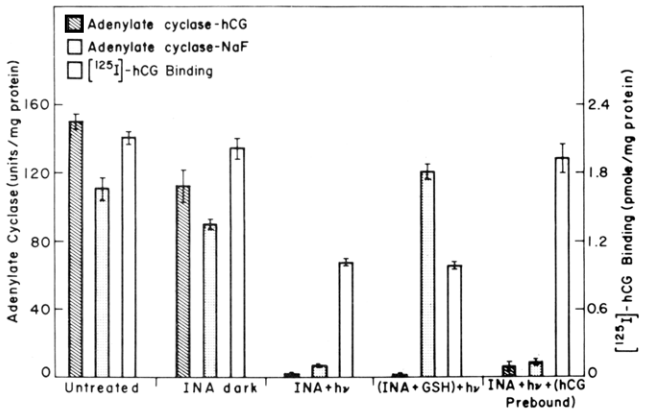

FIGURE 5: Effect of GSH and prebinding of hCG on inactivation by high concentration of INA. Membranes were incubated with (prebound) or without [^{125}I]hCG under conditions described for hormone binding. A sample from membranes incubated without [^{125}I]hCG was then put aside for further analysis (untreated). 1 mM INA (3000 nmol/mg of membrane protein) was then added to both membrane suspensions. All other details were as described in Figure 3. Adenylate cyclase activity and [^{125}I]hCG binding were then determined as described under Materials and Methods. [^{125}I]hCG binding was determined in all treatment groups except for the group that was already prelabeled with [^{125}I]hCG (prebound).

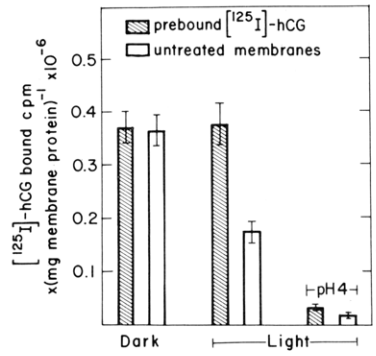

FIGURE 6: Effect of INA at high concentration on hCG binding. Membranes were incubated with [^{125}I]hCG under conditions otherwise used for determination of [^{125}I]hCG binding (prebound). Membranes were also incubated under the same conditions without [^{125}I]hCG (untreated). INA at a final concentration of 200 μM (600 nmol/mg of membrane protein) was then added as described under Materials and Methods. The amount of [^{125}I]hCG already bound or the residual binding activity was then determined in membranes that were not exposed to light (dark) or membranes that were irradiated (light). A sample of prebound membranes and a sample of untreated membranes to which [^{125}I]hCG was bound following irradiation were further incubated at pH 4.0 (sodium acetate, 0.2 M) for 5 min at 4 °C prior to filtration.

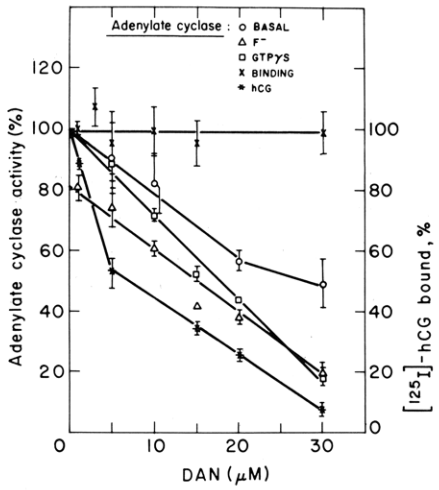

FIGURE 7: Influence of DAN on adenylate cyclase system. Membranes were treated with the indicated concentration of DAN as described under Materials and Methods. Values are expressed in percent of activity. 100% was taken as the activity of control membranes that were not treated with DAN. Adenylate cyclase activity and [^{125}I]hCG binding were performed as described under Materials and Methods. The effect of DAN on response of adenylate cyclase to hCG was calculated by subtracting the values obtained with 1 μM GTPγS from values obtained in the presence of 10 nM hCG + 1 μM GTPγS.

centrations of up to 50 nmol/mg of protein. Further increase in the concentrations of INA now gradually reduced the capacity of the membrane to bind [^{125}I]hCG. The maximal effect (50% inactivation) observed in these experiments was seen at INA concentration >200 nmol/mg of protein. These results clearly demonstrate the presence of at least three functionally different sites that may be affected by INA at distinctly different INA concentrations.

We next examined the action of INA on these three sites in the presence of GSH. In this experiment (Figure 5), membranes were pretreated with INA at 3000 nmol/mg of protein, and photolysis was performed in the presence or absence of 15 mM GSH. It was found that the inhibitory effect of INA on NaF stimulation was completely blocked in the presence of GSH. However, GSH did not prevent the inhibition of [^{125}I]hCG binding and the response of the enzyme to hCG as we have already seen (Figure 3).

Irradiation in the presence of high INA concentration of membranes to which [^{125}I]hCG was previously bound did not lead to dissociation of the bound hormone. One explanation for this result may be the posibility of covalent binding of the hormone to the receptor following light irradiation.

In order to test this possibility, native membranes and membranes to which [^{125}I]hCG was previously bound were subjected to treatment with INA. Binding of [^{125}I]hCG to the native membranes and the amount of [^{125}I]hCG already bound to prelabeled membranes were then determined (Figure 6). A sample of each membrane suspension was further incubated with acetate buffer at pH 4.0 (2 min at 4 °C). Treatment with buffer at this pH was previously shown to cause reversible dissociation of the hormone (Amir Zaltsman & Salomon, 1980). It was found that under these conditions bound [^{125}I]hCG dissociated from the membrane receptor even following INA treatment. This result proves that the stability of the [^{125}I]hCG receptor complex to treatment with INA does not result from covalent binding of the hormone to the receptor.

Effects of DAN on Adenylate Cyclase. The use of INA as a means of distinguishing between sites on the outer surface and the inner domain of the lipid bilayer of the membrane is based solely on the partition coefficient of this substance. It was therefore of interest to determine the action of DAN, an ionic derivative obtained as an intermediate in the synthesis procedure of INA. This material is insoluble in lipid and was regarded as a suitable analogue of INA that may affect the adenylate cyclase system only by interaction with sites exposed on the outer surface of the membrane. Membranes were incubated with increasing concentrations of DAN and subsequently exposed to light under conditions identical with those used in experiments with INA (Figure 2).

It was found (Figure 7) that, in contrast to INA, photolysis with DAN at concentrations up to 30 μM inactivates the adenylate cyclase in a nonselective manner. However, binding of [^{125}I]hCG to the membrane receptor was unaffected. No inhibitory effect of DAN could be observed in the absence of photolysis. Treatment with imidazole in excess, a procedure that results in the conversion of the diazo group into the imidazole–azo derivative, does not affect the inhibition by this

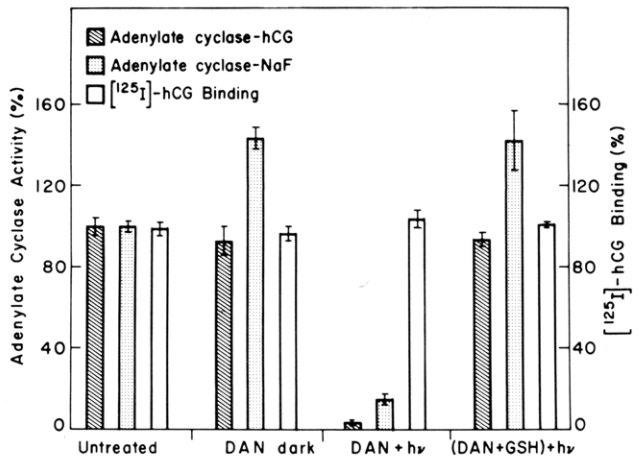

FIGURE 8: Effect of DAN on adenylate cyclase activity in the presence of GSH. Membranes were treated with DAN and GSH as described for INA in Figure 3, except that the DAN final concentration was 30 μM (90 nmol/mg of membrane protein). Residual adenylate cyclase activity in the presence of NaF or hCG and hCG binding were determined as described under Materials and Methods. 100% activity was that obtained with untreated membranes (control).

reagent of the cyclase activity. Inhibition is only observed in the presence of light. These results indicate that the reaction occurs through the photosensitive azide. Addition of 5-diazonionaphthyl 1-azide to the membranes under subdued light followed by centrifugation prior to irradiation gave inhibition nearly equivalent to that obtained in the absence of the centrifugation step. We now examined the hypothesis that DAN indeed acts at the aqueous phase of the membrane. Membranes were therefore incubated with 30 μM (90 nmol/mg of protein) DAN and subsequently exposed to light in the presence or absence of 15 mM GSH (Figure 8).

It was found that while DAN abolishes adenylate cyclase activity, GSH completely prevented the effect of DAN and protects the response of adenylate cyclase to NaF and hCG. Binding of hCG was unaffected by DAN. We also observed a slight stimulatory effect of DAN on the response of adenylate cyclase to NaF. This effect, however, seems to be unrelated to the photochemical process in question since it was seen also in the dark and was unaffected by GSH.

Discussion

The use of lipophilic reagents to identify the relative location of various components of membrane-bound enzymes and receptors requires a clear understanding of the sites within the membrane phase in which the reagents are confined. It is clear that the location of INA, or its photogenerated nitrene, and the site to which it will attach are dependent on the concentration of INA used. The high partition coefficient of INA ($>10^5$) (Bayley & Knowles, 1980) for the lipid phase of biological membranes implies that even low concentrations added to a cell or membrane suspension will almost quantitatively become incorporated into the membrane phase. If the lipid phase is present at concentrations of 1 μL/mL of the suspension, the final concentration of INA within the membrane phase will therefore be some 1000-fold greater than that calculated for the total volume of the reaction mixture. It appears likely, therefore, that at low concentrations of INA (0.02–10 μM), the ratio of membrane phospholipid to INA will be 100–1000 to 1 and the INA will be present within the "lipid core", that is, toward the center of the bilayer. The photogenerated nitrene would then be constrained within the lipid phase of the membrane. This is supported by the fact that the presence of 15 mM GSH during photolysis does not influence the inhibitory effect of INA on the coupling of the hormone receptor to the adenylate cyclase. It is at these concentrations that the most interesting results of this study are found.

Increasing INA concentrations to 100 μM and then to 1.0 mM will obviously lead to membrane phospholipid to INA ratios of 10/1 to 1/1. Clearly, at these concentrations every component of the membrane will becomes accessible to INA, and the photogenerated nitrene will in part exit from the bilayer and become accessible to the aqueous scavenger. The fraction of the nitrene that is scavenged by GSH is most probably labeling through the aqueous phase. It is therefore not surprising that INA at high concentrations is mimicked by its water-soluble analogue, DAN. DAN acts primarily at the lipid–water interphase and at low concentrations inhibits fluoride activation of adenylate cyclase. This effect can be completely prevented by GSH.

In all prior studies where INA was used as a radioactive photolabel, the concentrations of the reagent applied were less than 10 μM. Thus, it is not surprising that added GSH had no effect in the observed INA labeling pattern. The effects of low concentrations of INA on the adenylate cyclase system are interesting since they demonstrate that there is (are) a site(s) within the lipid bilayer that is (are) blocked by the covalent attachment of the nitrene. Function of this site is essential for the transfer of the signal from the hormone receptor to the regulatory guanine nucleotide binding protein (G/F) (Ross & Gilman, 1980). It should be emphasized that the reported effect of INA was not observed prior to photolysis. The covalent attachment of the nitrene to protein or lipid is required for the uncoupling to occur.

In contrast, the regulation of cyclase activity by G/F is not affected by INA as revealed by the unimpaired response of the enzyme to F^- ions and GTPγS. This result suggests, therefore, that the interphase between G/F and the catalytic unit (C) is probably not tightly associated with the lipid phase to which INA is confined at low concentrations. This assumption is further supported by the finding that GSH effectively protects the response of adenylate cyclase to F^- ions following treatment with high INA concentrations or low concentrations of DAN.

The finding that binding of the hormone to its receptor is insensitive to low concentrations of INA or DAN raises the possibility that either the receptor is not modified by nitrenes generated or else its modification does not result in loss of binding capability. The 50% loss of binding capacity as obtained at high concentrations of INA was not prevented by GSH. This result reveals a heterogeneous behavior of the receptor population, only part of which (50%) may after all be modified by photogenerated nitrenes from within the lipid bilayer.

The nature of the site of interaction of INA is presently unknown. It is of interest that other lipophilic reagents reactive in the apolar phase can similarly uncouple the receptor form the adenylate cyclase. Thus, in prior publications it was observed that lipophilic *N,N'*-dicyclohexylcarbodiimide (DCCD) could uncouple hormone response from the adenylate cyclase (Gitler et al., 1973; Schramm, 1976; Azulai & Salomon, 1980). These effects were observed at high reagent concentrations. Thus, 50% inhibition occurred at 6×10^{-5} M DCCD vs. 10^{-6} M for INA in the uncoupling of the response of adenylate cyclase to LH or hCG. In addition, DCCD affected hormone binding, but prior binding of the hormone prevented this effect. Both nitrenes and carbodiimides could react with sulfhydryls, amines, or phenol groups. Thus, it seems likely

that they interact with a site in the receptor or a complementary receptor binding site on G/F. The latter seems more likely since INA uncouples not only the hCG/LH receptor but also the response to follicle-stimulating hormone (FSH) (data not shown). It is also interesting to note that the addition of low INA concentrations followed by irradiation might lead to the generation of functionally uncoupled membranes or cells, which may be helpful in future analysis of the membrane-bound receptor system.

Acknowledgments

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Registry No. 5-Iodonaphthyl 1-azide, 63785-44-4; 5-diazonionaphthyl 1-azide, 88000-66-2; adenylate cyclase, 9012-42-4.

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Formaldehyde Metabolism by *Escherichia coli*. In Vivo Carbon, Deuterium, and Two-Dimensional NMR Observations of Multiple Detoxifying Pathways†

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ABSTRACT: ^{13}C NMR has been used to demonstrate the metabolism of dilute solutions of labeled formaldehyde by *Escherichia coli* to methanol, formate, carbon dioxide, and several other unidentified metabolites which contain labeled CH_2 groups. Aeration of bacterial suspensions within the spectrometer dramatically increased the rate of oxidation to formate and carbon dioxide. Deoxygenation with nitrogen gas virtually abolished all metabolism, as did the exposure of bacteria to very high formaldehyde concentrations. Deuterium NMR of whole cells in deuterium-depleted water further demonstrated the conversion of formaldehyde-d_2 to methanol-d_2, ruling out a formaldehyde dismutase as an important species. Two-dimensional proton–carbon chemical shift correlation was used to reveal the chemical shifts of the protons attached to ^{13}C labels in metabolites. The results indicate that formaldehyde is efficiently detoxified by the bacterial cell through a route or routes which do not appear to involve tetrahydrofolate. This detoxification may be in competition with the lethal antibacterial processes associated with formaldehyde.

Formaldehyde occurs in low concentrations in cells as the adduct N^5,N^{10}-methylenetetrahydrofolate, which is an important intermediate in the reactions of the C_1 pool. When tetrahydrofolate adducts are used as carriers, one-carbon units at the formate, formaldehyde, and methanol oxidation level are transferred to other molecules during biosynthesis (Benkovic, 1980). The major source of methylenetetrahydrofolate is the reaction between tetrahydrofolate and serine, catalyzed by serine hydroxymethyltransferase:

serine + tetrahydrofolate ⇌
glycine + methylenetetrahydrofolate

Since this reaction is reversible, it can also generate serine if methylenetetrahydrofolate is provided by another route such as the oxidative degradation of glycine. An alternative route to the adduct is direct chemical reaction of formaldehyde with tetrahydrofolate itself (Benkovic, 1978). Serine may subsequently be converted to pyruvate and, after decarboxylation and derivatization, to acetyl-CoA. In this way carbon atoms

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