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CHARACTERIZATION OF THE β_2-ADRENOCEPTOR-DEPENDENT ADENYLATE CYCLASE OF A431 EPIDERMOID CARCINOMA CELLS

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Abstract—In this study we characterize the β_2 adrenergic dependent adenylate cyclase system of epidermoid carcinoma cells (A431). (a) We show that the cells synthesize up to 130,000 [^{125}I]-cyanopindolol binding sites per cell when freshly plated, a value which decreased to 40,000–50,000 receptors/cell within 24 hr. Production of this high number of receptors can be strongly inhibited by actinomycin D. (b) We confirm and extend the fact that these β-adrenoceptors are of the β_2-subtype, using selective ligands (c), photoaffinity labeling with [^{125}I] CYP-diazirine identified two protein subunits: p59 and p72 (d), the β_2-adrenoceptor dependent adenylate cyclase desensitizes with half-life of 2.2 ± 0.3 min whereas the loss of [^{125}I]CYP binding from the cell surface requires longer exposure times to the agonist, (e) phorbol-12-myristate-13-acetate (PMA) has no effect on the desensitization process nor does it have any effect on the modulation of β-agonist affinity by guanyl nucleotides. Rather, PMA was found to stimulate adenylate cyclase activation by forskolin. We conclude that protein kinase C is probably not involved in the β-adrenoceptor desensitization in this cell line.

Recently, Strosberg *et al.* reported on the presence of a large number of β-adrenoceptors in the A431 epidermoid carcinoma cell line [1]. In their study, the authors used [^{3}H]-dihydroalprenolol and [^{3}H]CGP-1277 to measure receptor binding activity. Using catecholamines, they identified the receptor as a member of the β_2-subtype. In this communication we further characterize the β_2-adrenoceptors and the adenylate cyclase activity in these cells. In view of the increasing interest in the mode of β-adrenoceptor desensitization [2–4] and the suggestion that protein kinase C might be involved [5, 6], we examined the effect of the active phorbol ester phorbol-12-myristae-13-acetate (PMA)† on the process of β-adrenoceptor desensitization and its effect on the interaction between the GTP binding protein(s) and the β-adrenoceptor.

MATERIALS AND METHODS

Materials. ^{125}I-cyanopindolol (^{125}I-CYP) and ^{125}I-CYP-diazirine were obtained from Radiochemical Centre (Amersham, U.K.). Zinterol was obtained from Mead-Johnson (U.S.A.) and Trimethoquinol from Tanabe Seiyaku Co. (Osaka, Japan). Cycloheximide, α-amanitin and actinomycin D were obtained from Sigma. Other chemicals and biochemicals were of the highest purity available, usually from Sigma (U.S.A.) A431 cells were obtained from Dr J. Schlessinger (Weizman Institute of Science, Rehovot).

Growth of cells. A431 cells were plated at a density of 1×10^5 cells/well and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heated fetal calf serum. Cells were used 24 hr later in all experiments, except where indicated.

Binding of ^{125}I-CYP to intact cells. Binding experiments were conducted in 24-well Costar dishes on intact cells where the cell density was always between 2.5 to 3.0×10^5 cells/wells. Prior to the binding experiment, the cells were washed three times with Dulbecco's phosphate buffered saline (PBS) at room temperature. Binding was performed in PBS at 22° either in the presence or absence of 100 nM 1-propranolol at a final volume of 0.5 ml/well. Incubation was for 2 hr, after which the cells were washed three times with 2.0 ml cold PBS. The cells were then dissolved in 0.5 N NaOH with 0.1% SDS and ^{125}I counted in a gamma counter. Identical results were obtained when the ^{125}I-CYP binding was performed in DMEM. Scatchard analysis and displacement curves were analysed as described [7] and the values obtained represent the average of four independent experiments with standard error of the mean (SEM).

Binding of ^{125}I-CYP to cells removed from the dish. Cells were either removed by exposure to EDTA buffer (0.5 mM EDTA, 5 mM KCl, 135 mM NaCl, 4 mM $NaHCO_3$ and 5.5 mM glucose) for 30 min at 30° or by exposure trypsin-EDTA solution B (trypsin 1:250 in Pucks Saline A) for 60 sec at 30°. The cells were then centrifuged at 1000 *g* for 5 min, resuspended in DMEM and ^{125}I-CYP binding performed in 0.5 ml aliquotes containing 1.5 to 2.0×10^5 cells system at a concentration range of 20–150 pM ^{125}I-CYP. Non-specific binding was performed with 100 nM 1-propranolol.

Criteria for ^{125}I-CYP surface labelling. Equilibrium binding at 22° is complete within 30 min

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† Abbreviations used: CYP, cyanopindolol; DMEM, Dulbecco's modified Eagle's medium; IBMX, isobutyl-methyl-xanthine; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate.

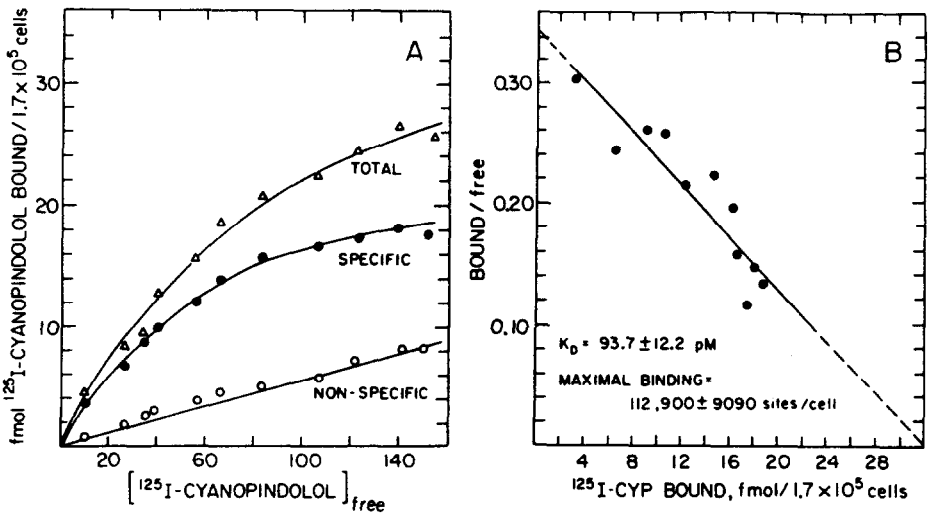

Fig. 1. Binding of ^{125}I-CYP to intact A431 cells: (A) data as obtained; (B) Scatchard analysis. Details are given in Materials and Methods.

($t_{\frac{1}{2}}$ = 8 min). In order to examine whether the ligand is partially internalized we examined the release of the ligand in to as described by Insel and his colleagues [8].

A431 membranes. Three types of membrane preparations were used—crude membranes, purified plasma membranes and shed vesicles. Crude membranes were prepared as follows. Cells were planted 24 hr before harvest on 10 cm Petri dishes. Then the cells were removed using an EDTA buffer at room temperature for 30 min. The cells were then centrifuged at 1000 *g* for 5 min. The cells were then homogenized in the cold using a tight fit glass homogenizer. The homogenate was centrifuged at 1000 *g* for 5 min at 4° to remove whole cells and nuclei. Supernatant was then centrifuged at 25,000 *g* for 15 min at 4°. The precipitate (P2) was resuspended in 50 mM Tris–HCl, 2 mM $Mg(Ac)_2$, 1 mM EDTA and kept in liquid nitrogen. 3×10^6 cells yield 1 mg membranes. Purified membranes were obtained according to Thom [9]; 1×10^7 cells yielded 1 mg membranes. Shed vesicles according to Cohen *et al.* [10]; 1.3×10^7 cells yielded 1 mg of shed vesicles membrane protein.

Binding of ^{125}I-CYP at A431 membranes. ^{125}I-CYP binding was performed essentially as previously described for NIH 3T3 cells [11] using 30–200 pM ^{125}I-CYP in the binding assay.

Binding of ^{125}I-CYP to membranes. The binding assay was performed at 30° in 50 mM Tris–HCl, pH 7.4, containing 2 mM EDTA, 1 mM $Mg(Ac)_2$, 20–100 μg protein/assay, in a final volume of 0.5 ml. The reaction mixture was incubated for 30 min, chilled in an ice water bath and filtered immediately onto a Linca binding apparatus, using Whatman GF/C glass filters. The filters were washed 3 times with 5.0 ml of ice-cold binding buffer and then counted in a gamma counter (Packard). Binding data was analyzed according to Scatchard using a SCAT computer program (see ref. 7).

Adenylate cyclase activity of intact A431 cells. Cells were labeled with 1 μCi [^{3}H] adenine/well for 2 hr in DMEM without serum. The cells were then washed three times with warm (37°) Dulbecco's PBS and once with warm DMEM. The cells were incubated with either 10 μM (−) isoproterenol or 1.0 μM 1-propranolol in the presence of 1.0 mM isobutyl-methyl-xanthine (IBMX), in a final volume of 0.5 ml DMEM at 37°. PMA or 4-β-phorbol alcohol were included at 100 nM whenever used. Parallel samples were incubated with either 10 μM forskolin, 10 μM forskolin plus 100 nM 4-β-phorbol, or 10 μM forskolin and 100 nM PMA for 5 min. At different time points, 0.4 ml ice-cold 25% trichloroacetic acid was added, followed by 0.1 ml of a mixture containing 0.5 mM of the following ingredients (final concentrations): adenosine, adenine, 5′-AMP, 5′-ADP, 3′-, 5′-cAMP, 0.5 mM of each; 5.0 mM ATP and 3000 [^{32}P]cAMP/well. The sample was then analyzed for cAMP according to Salomon *et al.* [12].

Photoaffinity labeling using ^{125}I-CYP-diazirine. Intact A431 cells were photoaffinity labeled by ^{125}I-CYP-diazirine as described [13]. Further details are given in the legend to Fig. 6.

RESULTS

Binding of ^{125}I-CYP to intact cells

Binding of ^{125}I-CYP can be measured directly on intact cells either on the tissue culture dish (Fig. 1) or in suspension subsequent to the removal of cells from the dish (Fig. 2) which express 50,000 ± 3000 receptors per cell when plated at a density of 1×10^5 cells/well and grown for 24 hr prior to the binding experiment. When plated at 2×10^4 cells/well, the number of β-adrenoceptors/cell rises to 130,000/cell at 4 hr subsequent to seeding but falls off to about 50,000 receptors/cell within 24 hr (Fig. 3). The number of receptors/cell measured is the same whether the measurement is on attached cells or on cells which have been detached from the culture dish by either EDTA or trypsin-EDTA. The K_D for ^{125}I-CYP is between 42 and 118 pM for intact cells (Fig.

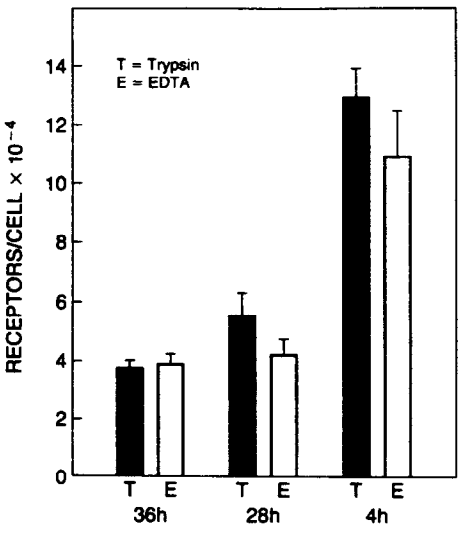

Fig. 2. Binding of ^{125}I-CYP to detached A431 cells. Cells were grown as described but detached from the dish either by EDTA alone or by trypsin-EDTA as described in Materials and Methods. Maximal ^{125}I-CYP binding was obtained from Scatchard plots and the values represent the average of three independent binding experiments with SEM.

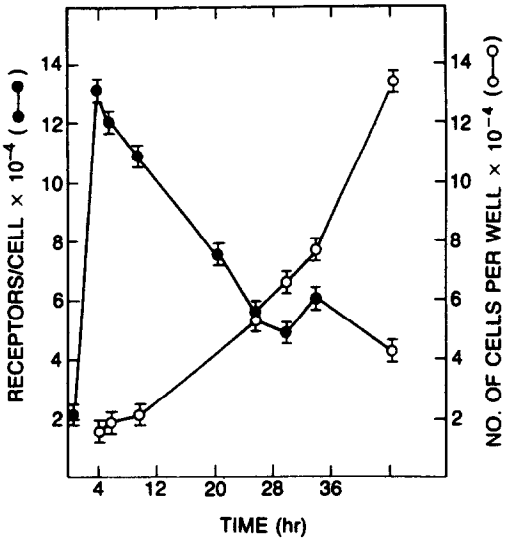

Fig. 3. The number of β-adrenoceptors/cell in A431 cells as a function of time subsequent to seeding. Cells were seeded at a density of 2×10^4/well, as described, and ^{125}I-CYP binding was measured at the given intervals as described in Materials and Methods.

1), and for different membrane preparations (Table 1), a slightly higher value than the typical range of 30–60 pM.

The effect of inhibitors of protein synthesis and nucleic acid synthesis on the number of β-adenoceptors

When either cycloheximide, α-amanitin, or actinomycin D are included in the medium, the sharp increase in the number of β-adrenoceptors in the first 4 hr subsequent to seeding is inhibited. In the presence of actinomycin D the number of receptors increases from 22,000 to only 59,000 whereas in its absence the number of receptors/cell can increase up to 120,000 (Fig. 4).

Binding of ^{125}I-CYP to membranes

Three types membrane preparations from A431 cells were used to examine ^{125}I-CYP binding. Table 1 summarizes the results obtained from these measurements. The measured values correspond well to the measurements of ^{125}I-CYP on the intact cells.

The release of ^{125}I-CYP from the cell surface

When ^{125}I-CYP binding is conducted at 22° 85 ± 4% of the ligand can be removed by washing the cells with an acidic buffer: 0.5 M NaCl/0.2 M Acetic acid pH 2.5 by the protocol described by Mohan *et al.* for S49 cells [8].

Pharmacological characteristics of the β-adrenoceptor

The β-adrenoceptor on A431 cells belongs to the β_2-adrenergic type, since β_2-specific ligands displace ^{125}I-CYP more effectively than β_1-adrenergic ligands (Fig. 5). Moreover, the photoaffinity label ^{125}I-CYP-diazirine labels two protein bands, p59 and p73, much higher than the p43 characteristic of the β1-adrenoceptor of turkey erythrocytes (Fig. 6).

Adenylate cyclase activity of the intact cells and its desensitization

When intact A431 cells are challenged with (−)-isoproterenol, a linear response is obtained for the first 1–2 min, when the response becomes attenuated

Table 1. ^{125}I-CYP binding to A431 membranes

Type of membrane preparation	Maximal ^{125}I-CYP binding (fmol/mg)	Dissociation constant (pM)	Number of receptors/cell§
Crude (P2)*	163 ± 12	56 ± 8	32,000
Purified†	311 ± 24	118 ± 18	62,000
Shed vesicles‡	255 ± 22	42 ± 9	51,000

* This study.
† According to Ref. 9.
‡ According to Ref. 10.
§ Calculated from the conversion factor number of cells/mg protein (see Materials and Methods).

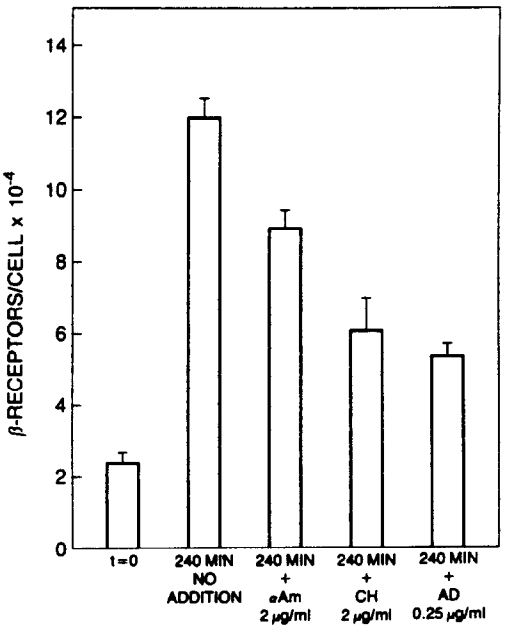

Fig. 4. The inhibition of β-adrenoceptor synthesis. Cells were seeded at a density of 2×10^4 cells/well in the absence or the presence either 2.5 μg/ml α-amanitin, 2.0 μg/ml cycloheximide or 0.25 μg/ml Actinonycin D. After 4 hr the number of receptors by conducting a ^{125}I-CYP binding experiment on the intact cells. The data present the maximal ^{125}I-CYP binding obtained from three independent saturation experiments plus the SEM.

with half-life of $t_{\frac{1}{2}} = 2.0$–2.5 min (Fig. 7). If isoproterenol is removed from the medium 6 min after its application no loss of ^{125}I-CYP binding sites can be observed. Forskolin stimulation remains linear. Exposure to (−)isoproterenol for 30 min leads to a reduction of ≥85% in the number of measurable ^{125}I-CYP binding sites (Fig. 8) and 68 ± 5% loss of isoproterenol dependent adenylate cyclase activity (Fig. 7).

The effect of PMA

Preincubation of intact cells for 3 hr with up to 100 nM PMA did not induce, by itself, the desensitization of β-receptor-dependent adenylate cyclase, nor did it alter the time course of (−)isoproterenol-induced desensitization. Rather, pretreatment with 100 nM PMA enhanced the adenylate cyclase activity (Fig. 7) stimulated by either isoproterenol or forskolin. The inactive 4-β-phorbol had no enhancement effect (Fig. 7). Pretreatment with 1.0 nM or 10 nM PMA did not have any effect on the (−)isoproterenol-induced stimulation and desensitization (data not shown). Subsequent to 2 hr of treatment with 100 nM PMA, no change in the number of β-adrenoceptors was noted or a change in the affinity of the receptor toward the ^{125}I-CYP (Fig. 8).

PMA does not affect the decrease in agonist affinity by guanyl nucleotides

When the binding of β-agonists was measured in the presence and absence of GppNHp, the well-recognized [14] pattern of reduced agonist affinity is seen (Fig. 9). Pretreatment of the cells with PMA does not seem to modulate the GppNHp effect on β-agonist affinity nor does it affect the affinity of the β-receptor toward isoproterenol in the absence of added GppNHp.

DISCUSSION

The A431 system

This study confirms and further extends the original observation [1] that the A431 epidermoid carcinoma cell line may be a useful system to study the β_2-adrenoceptor dependent adenylate cyclase

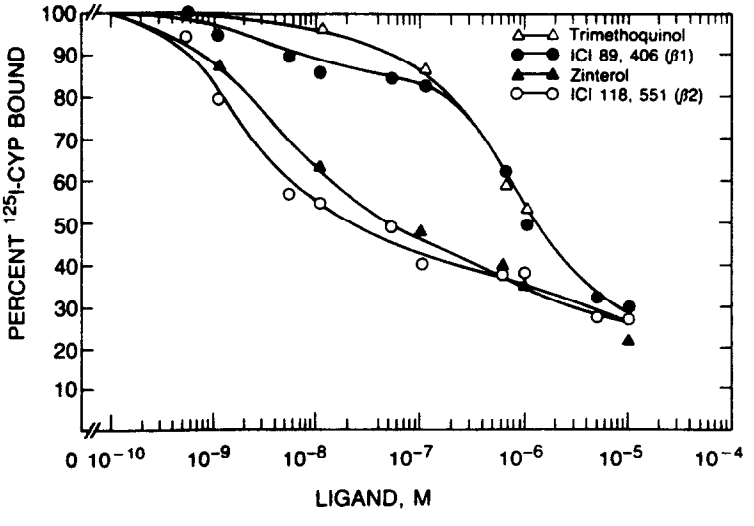

Fig. 5. The β_2 nature of the A431 receptor. Binding experiments were conducted as in Fig. 1, using a single ^{125}I-CYP concentration (110 pM) and increasing concentrations of the competing ligand. The data is depicted as percent of total ^{125}I-CYP binding. It can be seen that the non-specific binding is 28 ± 2.5% of the total.

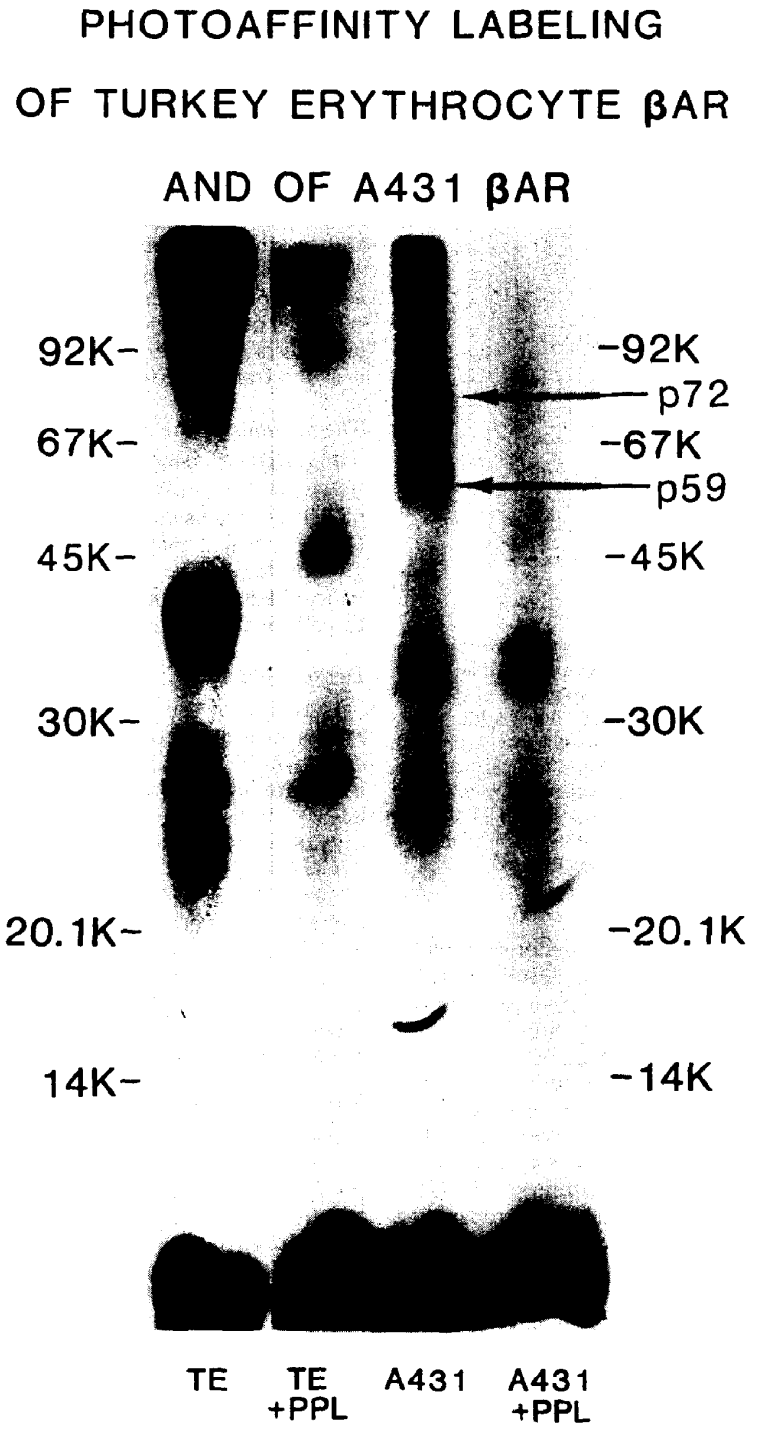

Fig. 6. Identification of the β-receptor protein subunits in A431 cells. Cells were labeled exactly as described by Burgermeister *et al.* [9]. Cells or turkey erythrocyte membranes (0.1 mg/ml) were incubated in Dulbecco's PBS in the presence of 0.1 nM ^{125}I-CYP-diazirine, and u.v. irradiated, as described, for 30 min at 0° [9]. Subsequent to irradiation, the cells were extensively washed, dissolved in boiling electrophoresis sample buffer and electrophoresed according to the Laemmli procedure [16].

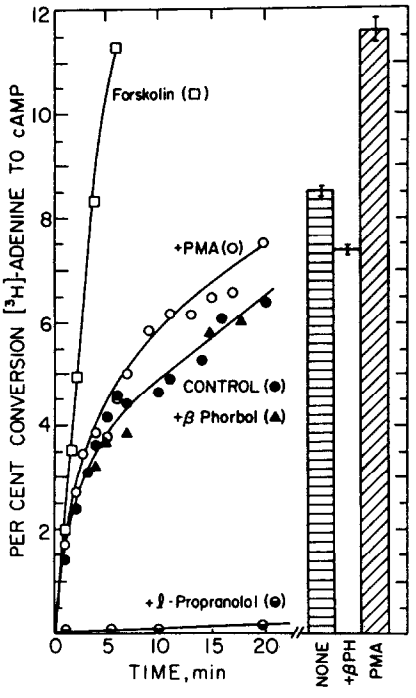

Fig. 7. Adenylate cyclase activity and its densitization in intact A431 cells. The concentrations of PMA and 4-β-phorbol were 100 nM. The final concentration of *d,l*-propranolol, *l*-isoproterenol and forskolin, when added, was 10 μM and 100 μM, respectively. All other experimental details are given in the text. The forskolin-induced accumulation of [³H]cAMP at a 4 min time point is shown in the histogram on the right-hand side of the figure. The effect of pre-incubation with PMA and β-phorbol for 15 min at 37° in Dulbecco's PBS is also depicted.

system. Using specific β_1 and β_2 adrenergic ligands, we characterize these receptors as β_2-subtype (Fig. 5) although the presence of some β_1-adrenoceptors cannot be excluded because of the non-Michaelian shape of the displacement curves. We also show that ^{125}I-CYP can be successfully used to probe the receptors on the intact cells (Figs 1–3). The measurement of ^{125}I-CYP binding can be performed either directly on the dish (Figs 1 and 3) or in suspension on detached cells (Fig. 2). Furthermore, ^{125}I-CYP binding on three types of membrane preparation corroborate the findings on intact cells (Table 1). When the ^{125}I-CYP binding experiments are conducted at 22° most of all the ligand remains in the cell surface whereas at 37° there is some internalization (Table 2). The very high number of receptors reported by Strosberg and coworkers [1] is essentially confirmed, but we show that the number of receptors per cell is highest shortly after seeding.

Inhibition of β-adrenoceptor synthesis

The high increase in the number of β-adrenoceptor subsequent to seeding (Fig. 3) is strongly inhibited in the presence of actinomycin D or cycloheximide and less so by α-amanitin (Fig. 4). These results suggest that the rapid increase in the number of β-adrenoceptors within the first 4 hr is due to *de novo* synthesis of receptors. The observation that actinomycin D is most effective suggests that the inhibition is at the transcription level.

The subunits of the β_2-adrenoceptor

Photoaffinity labelling of the A431 β-adrenoceptor from A431 cells reveals two protein bands p59 and p73. These bands are relatively wide on the SDS gels (Fig. 6), which may indicate that these represent glycoproteins. The appearance of two bands may indicate a proteolytic degradation of the p72 protein to p59, or two forms of glycosylated receptor.

Densensitization

Exposure of the A431 cells to (−)isoproterenol results in a rapid desensitization of these cells to this ligand. The long term (30 min) decrease in the adenylate cyclase response (Fig. 7) occurs concomitantly with the loss of ^{125}I-CYP binding (Fig. 8); up to 85 ± 3% of the ^{125}I-CYP binding is lost from the surface of the cells upon exposure to (−)isoproterenol. Long term (30 min) loss of hormone stimulated adenylate cyclase activity reaches 68 ± 5%. Within the first few minutes of exposure to (−)isoproterenol only receptor to adenylate uncoupling takes place with no loss of receptors. This observation confirms results of other groups on different cell lines (for reviews see refs 2 and 3).

The effect of phorbol ester

PMA and its active analogs induce the activation of protein kinase C and, thereby, the phosphorylation of target proteins which are involved in key metabolic processes [15]. It was recently suggested that protein kinase C is a key element in the physiological mechanism for the β-adrenoceptor

Table 2. The release of bound ^{125}I-CYP

Temperature	Time of incubation (min)	fmoles ^{125}I-CYP bound per well	^{125}I-CYP released
22°	30	6.5 ± 0.2	5.8 ± 0.1
37°	30	8.2 ± 0.1	5.8 ± 0.2

Binding was performed as described in Materials and Methods using 38 pM ^{125}I-CYP with cells, which were seeded 24 hr earlier at a density of 1.2×10^5 cells/well.
Release of ^{125}I-CYP was induced by removing the medium, washing three times with ice-cold PBS and then treating with 0.5 M NaCl/0.2 M acetic pH2.5 acid for 15 min, at 2°, as described [8].

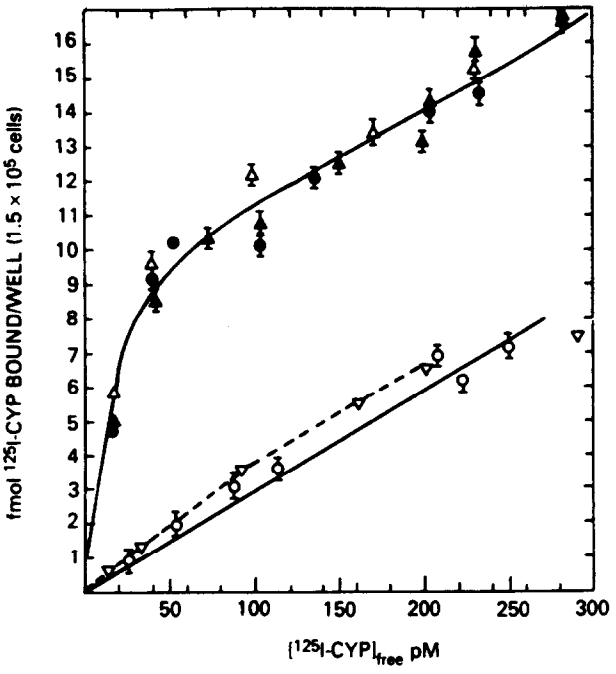

Fig. 8. Binding of ^{125}I-CYP to isoproterenol desensitized cells and cells treated with PMA. The medium was replaced with DMEM without serum, supplemented either with 100 nM PMA or 100 nM 4-β-phorbol (inactive), and the cells were incubated for 2 hr at 37° in the incubator. In parallel, cells were incubated with 10 μM(−)isoproterenol at 37° for 30 min. Subsequently, the cells were washed three times with DMEM at 37° and twice with Dulbecco's PBS at 25° and then subjected to a 2-hr binding experiment in DMEM as described in Fig. 1 and in Materials and Methods. Total ^{125}I-CYP binding: ●—●, intact untreated cells; ▲—▲, PMA treated (100 nM) cells; △—△, cells treated with 4-β-phorbol 100 nM; ▽—▽, cells treated with 10 μM (−)isoproterenol for 30 min at 37° in DMEM. Non-specific binding (○—○) was conducted in the presence of 100 nM (−)propranolol. Under the washing conditions described, all the isoproterenol is completely removed. When 800,000 cpm [^{3}H]isoproterenol with the 10 μM(−)isoproterenol is used, less than 65 cpm remain after the third washing. It should be noted that this washing protocol also removes >98.6% bound ^{125}I-CYP from cells [24]. The non-specific binding is 28 ± 1% at 110 pM ^{125}I-CYP. The displacement curves of Zinterol and ICI 118,551 possess a Hill coefficient of n_H ± 0.88 + 0.1 and therefore closely Michaelian (non-cooperative).

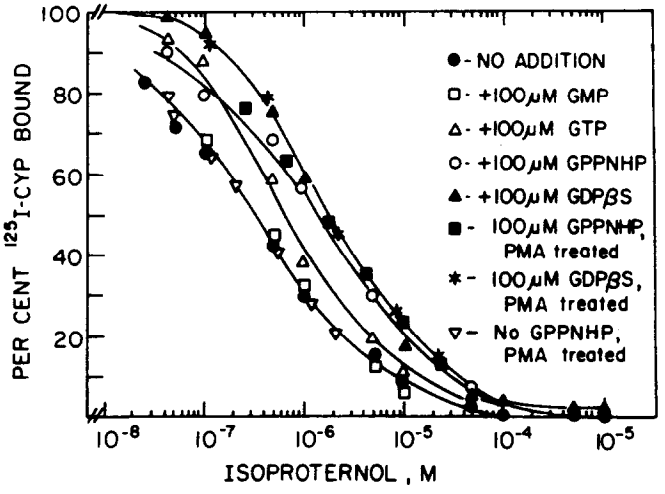

Fig. 9. The effect of PMA pretreatment on the affinity of the β-receptors toward agonists. Cells were treated with 100 nM PMA, as described in Fig. 6. Cell homogenates and membrane fractions were prepared as previously described [1]. ^{125}I-CYP binding was measured in the presence of 120 pM ^{125}I-CYP with or without 10 μM *d,l*-propranolol, as described in Materials and Methods. The non-specific binding was 28 ± 1% of the total ^{125}I-CYP binding. 100% refers to maximal specific ^{125}I-CYP binding (0.35 pmol/mg). The concentration of ligands included in the displacement experiments is depicted in the figure.

desensitization [5, 6]. Indeed, it was demonstrated that PMA can cause up to a 40% decrease in the β-adrenoceptor dependent adenylate cyclase activation in duck erythrocytes [6] and turkey erythrocytes [5], concomitantly with the phosphorylation of the receptor. Our findings demonstrate that PMA neither induces β-receptor densensitization in A431 cells nor does it enhance the (−)isoproterenol induced desensitization. Also, PMA treatment does not alter the mode of interaction of the β-receptor with the stimulatory GTP binding protein G_s. We conclude this from the observation that the reduction in β-agonist affinity by guanyl nucleotides is identical in untreated A431 cells and in PMA treated cells (Fig. 9). However, since we know that under the same experimental conditions which we applied to the A431 cells PMA induced EGF receptor phosphorylation [16–18], internalization and recycling [19], we have reason to believe that PMA has reached its intracellular physiological targets. Recent experiments on lymphoma S49 cells [21], rat liver cells [22] and rat myoblasts [23] reveal that phorbolesters do not affect the β-adrenergic response and, as in the present report (Fig. 7), augmentation rather than inhibition of the β-adrenergic response is noted. Thus it seems that in cells which exhibit the capacity to desensitize the β-adrenergic ligands, protein kinase C is not involved in the process. The behavior of liver cells [22], S49 cells [21] and rat myoblasts [23] as well as A431 cells is different from that of avian erythrocyte β-adrenoceptor dependent adenylate cyclases, where phorbol ester was found to enhance desensitization [5, 6]. The enhancement of adenylate cyclase activity by PMA observed in the four cell types cited may be at the level of the GTP binding proteins or the catalytic unit itself, since the forskolin dependent adenylate cyclase activity is also enhanced in both the S49 cells [21] and the A431 cells (Fig. 7). The unaltered effects of guanyl nucleotides on β-agonist binding, subsequent to PMA pretreatment (Fig. 9), demonstrates that even if the β-adrenoceptor is phosphorylated after PMA pretreatment, its modification does not modify its mode of coupling to the GTP binding protein(s) which are coupled to adenylate cyclase.

The different results obtained with the four cell types cited as compared to those obtained for turkey erythrocytes may be due to the dissimilar physiological make up of avian erythrocytes: (a) Intact turkey erythrocytes do not display homologous desensitization towards β-agonists even after 45 min of exposure to β-agonists [24], nor do they lose β-receptors from their surface upon prolonged exposure to β-agonists [25] as do most cells possessing β-adrenoceptors [2, 3]. However, heterologous densensitization has been found to occur [26]. (b) Avian erythrocytes are deficient in a number of biochemical features and lack certain cellular organelles [27] such as coated pits which are involved in receptor down regulation [28].

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