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CHARACTERIZATION OF THE BETA-ADRENERGIC RECEPTORS OF CULTURED HUMAN EPIDERMAL KERATINOCYTES

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Abstract—The presence of β-adrenergic receptors has been demonstrated in membrane preparations from passaged human epidermal keratinocytes. The receptors were characterized in terms of density and binding properties. Using the tritiated β-adrenergic antagonists dihydroalprenolol and propranolol, the equilibrium dissociation constant (K_d) was found to be about 1.4 nM for the two antagonists with a receptor density of approximately 280 fmol/mg membrane protein. Stereospecificity of the binding sites was shown by the much lowered affinity to D-isoproterenol as compared to that of L-isoproterenol. By the use of subtype specific antagonists, the receptors were classified as β_2 adrenoceptors. This finding is supported by the relative order of affinities of the agonists isoproterenol > epinephrine > norepinephrine. The K_d value for dihydroalprenolol was approximately the same when determined from equilibrium binding studies or from association and dissociation kinetics, suggesting that the ligand binding is a single step bi-molecular reaction.

The control of epidermal cell proliferation and differentiation is thought today to be modulated, at least in part, by cyclic AMP, the second messenger of β-adrenergic stimulation [1, 2]. Although the function of catecholamines as modulators of epidermal processes is by no means established, their possible involvement in such a role has been repeatedly implicated.

Bullough and Lawrence [3] have first suggested a possible correlation between serum epinephrine levels and the diurnal variations of epidermal mitotic activity. More direct evidence was brought forth by Powell *et al.* [4] who demonstrated that the addition of β-adrenergic agonists to isolated epidermal sheets resulted in an increased level of intracellular cAMP†, followed by a reduction in the number of observed mitoses and the suggestion was made [5] that a defect of the cAMP cascade may be responsible for the hyperproliferative disorders seen in psoriasis. This effect of β-adrenergic stimulators could be mimicked by the addition of cAMP and theophylline (a phosphodiesterase inhibitor), to mouse epidermis *in vitro* [6], which resulted in an inhibition of cell mitotic activity similar to that achieved by β-adrenergic agonists. The opposite effect was obtained by treatment with β-adrenergic antagonists such as propranolol, which was reported to cause hyperproliferative skin changes in guinea pig and man [7, 8], assumed to be related to lowering of the intracellular cAMP levels.

Whereas the studies on mammalian epidermis, both *in vivo* and *in vitro*, indicate a growth inhibiting effect of cAMP elevating compounds, the results on cultured epidermal keratinocytes are conflicting and range from those showing growth inhibition and induction of differentiation, through studies reporting no effects due to changes in the levels of intracellular cAMP, and to those showing cell growth stimulation by cAMP elevation [9–11].

A considerable number of publications offer indirect evidence for the involvement of β-adrenergic receptors in epidermal regulatory processes. We are aware, however, of only two reports that have tried to demonstrate directly the presence of β-adrenergic receptors in skin cell membranes [12, 13] and both have used mouse epidermal cells as their source for the membranes.

The possible involvement of β-adrenergic stimulation in the development of hyperproliferative skin disorders, the conflicting results on the action of β-adrenergic effectors on cultured keratinocytes, and the lack of information from binding studies with isolated membrane preparations from human skin cells have stimulated us to characterize the β-adrenoceptor population on the cell membrane surface from cultivated human keratinocytes by means of equilibrium binding and competition studies as well as by association and dissociation kinetics.

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† Abbreviations used: cAMP = Cyclic 3′,5′-adenosine monophosphate; EGF = Epidermal growth factor; EDTA = Ethylenediaminetetraacetic acid; PBS = Phosphate buffered saline containing no Mg^{2+} or Ca^{2+} ions; DHA = L-Dihydroalprenolol; DPM = Decompositions per min.; Tris = Tris(hydroxymethyl)aminomethane.

MATERIALS AND METHODS

Preparation of keratinocytes

Human breast skin from plastic surgery and infant foreskin has been used as the sources for keratinocytes. Thin split-thickness skin sections were obtained by the use of a Castroviejo keratotome set at a depth of 0.3 mm. Epidermis and dermis were separated after treatment with 0.3% (w/v) trypsin (Gibco, 1 : 250) for about 1 hr at 37° as described by Liu and Karasek [14]. Dermal and epidermal flaps were agitated gently in growth medium to liberate basal and malpighian cells. The liberated cells were collected by centrifugation at 800 *g* for 5 min. They were then seeded either at high density (5×10^4 translucent cells/cm^2) on collagen coated dishes according to the method of Liu and Karasek, or at low density (5×10^3 cells/cm^2) together with UV-irradiated mouse 3T3 fibroblasts (ATCC line CCL92; 1.6×10^4 cells/cm^2) on regular plastic tissue culture dishes according to the procedure of Rheinwald and Green [15]. The cells were grown to confluency in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 1 μM hydrocortisone, 100,000 units/l penicillin and 100 mg/l of streptomycin. When the 3T3 feeder cell technique was used, the medium was additionally supplemented with 0.1 nM cholera toxin and 1.6 nM EGF [16]. Most of the feeder cells were detached from the plastic support during expansion of the keratinocyte clones and were removed with the routine medium changes.

Normally, the cells were subcultured in one or two passages before being used for the preparation of plasma membranes. When necessary, the cells were selectively decontaminated from residual feeder cells by incubation with 0.02% EDTA and were then harvested by scraping with a rubber policeman or by digestion with 0.05% trypsin. The harvested cells were stored at − 80° in the culture medium supplemented with 10% (v/v) glycerol.

In the binding experiments reported below, no difference in receptor content or characteristics was found between cells from adult or infant donors or between the two cultivation methods.

Preparation of plasma membranes

Homogenates of the cells were prepared by the nitrogen decompression technique [17, 18]. The cells were washed 4 to 5 times by centrifugation for 5 min at 1000 *g* with resuspension in five volumes of PBS. The washed cells were suspended in three volumes of PBS and placed in the Parr Cell Disruption Bomb (Parr Instrument Co., Moline, IL, U.S.A.). The cell suspension was then pressurized with nitrogen to a pressure of 1.38×10^7 Pa (138 bar) for 10–20 min until cells and solution were in equilibrium with the pressurized gas. Disruption was achieved by allowing the cell suspension to flow out through a needle valve, whereby an instantaneous decompression resulted. After two cycles of pressurizing and decompression, no whole cells could be detected by light microscopy.

Plasma membranes were collected by centrifuging the cell homogenate at 100,000 *g* for 45 min at 4° and washing the pellet three times by centrifugation and resuspension. The washed membranes were taken up in Tris-HCl buffer, pH 7.8 (for composition see below), to yield an approximate protein concentration of 3 mg/ml. This type of membrane suspension was employed for all the binding studies and was kept frozen in small portions in liquid nitrogen until used.

Binding assay

Prior to performing the binding experiment, the membranes were thawed and homogenized in a loose neck Potter–Elvehjem homogeniser, so that an evenly dispersed membrane preparation was obtained. Binding to the β-adrenergic receptors was carried out at 25° in a total volume of 0.25 ml in Tris-HCl, pH 7.8, consisting of 0.05 M Tris; 0.12 M NaCl; 0.005 M KCl and 0.025 M $MgCl_2$. All solutions of ligands and competitors were made in this buffer. When the binding of isoproterenol, epinephrine or norepinephrine was measured, the buffer contained in addition 1 mM L-ascorbic acid (which had no effect on the binding by itself) to prevent oxidation of the catecholamines. The binding assay was started by the addition of the membrane suspension to the incubation mixture and the incubation was carried out for 40 min to ensure binding equilibrium.

At the end of the incubation time, two aliquots of 0.1 ml each were taken from each tube and diluted with 5 ml of ice-cold Tris buffer. The diluted samples were immediately filtered through Whatman GF/C glass fiber filters under suction and the filters were washed twice with 5 ml portions of ice-cold Tris buffer. The filters were dried at 110° for 30 min, placed in 10 ml of scintillation cocktail (Picofluor scintillation cocktail, Packard, U.S.A.) and the absorbed radioactivity was counted in a liquid scintillation spectrometer, using the external standard method to correct for quenching and to calculate DPM. Specific binding [19, 20] was defined as the amount of radioligand retained by the filters in the absence of a competing ligand (total binding), minus the amount of radioligand bound in the presence of 5 μM propranolol or alprenolol (non-specific binding). The total concentration of the labeled ligand was determined by counting a 0.02 ml sample of the incubation mixture prior to filtration.

To determine the binding of unlabeled ligands, their potency as competitors of [^{125}I]-iodocyanopindolol was measured. This ligand was chosen since it was previously shown [21] to have a very high affinity to the β-adrenergic receptors, to have no affinity towards either α-adrenergic or 5-HT receptors and to show no discrimination between the β_1 and β_2 adrenergic receptors. The high specific activity of the iodine allows in this case accurate measurements also at high competitor concentrations, when the amount of bound radioactive ligand is minimal. In this type of study, the membranes were incubated with a constant concentration of [^{125}I]-iodocyanopindolol and increasing concentrations of the non-radiolabeled competitor ligand. The binding of the iodinated antagonist under these conditions was measured as described above for saturation binding (using a gamma counter instead of liquid scintillation counter).

The kinetics of the binding to the β-adrenergic receptors was studied in two separate types of

experiments. To measure the dissociation reactions, membranes were equilibrated with 2–3 nM of ^{3}H-DHA for 40 min to achieve equilibrium conditions (at 25°). The dissociation reaction was started by the addition of a large excess of unlabelled alprenolol (approximately 1000–10,000 fold molar excess) and the amount of the bound tritiated ligand was measured as a function of time as described above. The association reaction was measured at 25° using essentially the same technique as for measuring the binding at equilibrium, but using varying incubation times ranging from 1 to 40 min. For both the association and dissociation experiments, an incubation volume of 3 ml has been used, so that the reaction could be followed with the same incubation mixture.

The experimental results were computer analysed with the help of several algorithms for non-linear regression analysis. For saturation binding and competition studies, the appropriate forms of Clark's equation [22] were employed as the regression model. For the analysis of total binding in saturation experiments, the non-linear regression model included a linear component, corresponding to the non-specific binding, while in the analysis of competition experiments, the non-specific binding was a constant value (iterated or measured), corresponding to the binding of the iodinated ligand at the highest competitor concentrations. In addition, Scatchard transformation of the binding data was performed [23], to obtain a linearized function which was then analysed by a linear regression algorithm.

The association reaction was analysed by the "NONLIN" algorithm of Metzler *et al.* [24], using as a regression model the differential equation describing the rate of association (see legend to Fig. 5(b)). This equation was numerically integrated by the curve fitting algorithm.

Chemicals

[^{3}H]-L-Dihydroalprenolol (^{3}H-DHA), (49.4 Ci/mmol), [^{3}H]-L-propranolol (28.7 Ci/mmol) and [^{125}I]-iodocyanopindolol (specific activity of 2000 Ci/mmol or higher) were purchased from New England Nuclear Co. Radiochemical purity was checked by thin layer chromatography using the solvent systems recommended by the manufacturer. EGF was a product of Collaborative Research (Waltham, Ma., U.S.A.). Cholera toxin, L-alprenolol, DL-propranolol, L- and D-isoproterenol, L-epinephrine and L-norepinephrine were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The ligands practolol and ICI 118 551 were a gift from ICI-Pharma, Enghien les bains, France; Metoprolol was obtained from Ciba-Geigy Co., Rueil Malmeison, France. Betaxolol (SL 75 212) and IPS 339 were a gift from Dr. S. Z. Langer, L.E.R.S., Paris, France; DL-Salbutamol was a gift from Dr. C. Hensby (CIRD). All other chemicals were of the finest grade available and were used as obtained. Protein was determined according to the method of Lowry [25], using human serum albumin (Cohn fraction V, Sigma) as the standard.

RESULTS AND DISCUSSION

In attempting to homogenize the harvested keratinocytes, techniques such as a motor-driven teflon-glass homogenizer, Polytron homogenizer and glass–glass homogenizers (in combination with hypotonic buffers and/or several freeze-thaw cycles) were found to damage the cells and to release most of the cytoplasmic content, but when observed under the microscope, most cells appeared to have maintained their shape and many were still nucleated. These preparations showed a high non-specific binding and were considered inappropriate for the study of binding to membrane receptors. The nitrogen cavitation technique, employing a pressure of 138 bar of nitrogen, was the only method found to completely disrupt the cells and to yield a membrane preparation free from cell ghosts (Fig. 1).

The binding of the β-antagonist ^{3}H-DHA to keratinocyte membranes is shown in Fig. 2. The non-specific binding, measured in parallel in the presence of 5 μM DL-propranolol, is linear over the entire concentration range employed, is non-saturable and is less than 30% of the total binding at the highest DHA concentrations. Control experiments showed that most of the non-specific binding takes place on the membranes and less than 20% is bound to the glass fiber filter. To estimate the non-specific binding, several concentrations of competitor (propranolol) were tried, ranging from 50 μm to 0.1 μM. At the lower concentrations the non-specific binding curves obtained showed a downward curvature, indicating an incomplete competition at the receptor site. Mathematical analysis of the total binding curves yielded a value for the non-specific binding which was very close to that obtained with 5 μM propranolol and this concentration was then employed routinely to evaluate the non-specific binding.

Analysis of the total binding curve yielded a value of (1.26 ± 0.17 SD) nM for the equilibrium dissociation constant (K_d) and (268 ± 20 SD) fmol/mg membrane protein as the receptor concentration. The analysis of the specific binding (obtained as the difference between the total and the non-specific binding curves) yielded a K_d value of (1.36 ± 0.12 SD) nM and a receptor concentration of (282 ± 9 SD) fmol/mg membrane protein. These results can be regarded as being identical to those obtained from the analysis of the total binding curve. Figure 3 shows the linearized plot of the specific binding curve after Scatchard transformation of the data. K_d and receptor concentration are essentially the same as those obtained from the analysis of the untransformed data. Attempts to fit the data to a different model, assuming two different receptors or an interacting receptor population (cooperative effects) did not improve the fitting of the data points to the calculated binding curve and statistical analysis of the fittings to the various models [26], suggested the higher probability of a binding model assuming a single, homogeneous receptor population. This assumption is supported by the linearity of the Scatchard plot as well as by Hill's plot of the data (Fig. 4), which yields a linear curve with a Hill coefficient of unity, indicating a uniform and non interacting receptor population and a K_d value of 1.36 nM, in complete agreement with the previous analysis. Similar results were obtained when tritiated L-propranolol was used as the labeled ligand and L-alprenolol as the competitor.

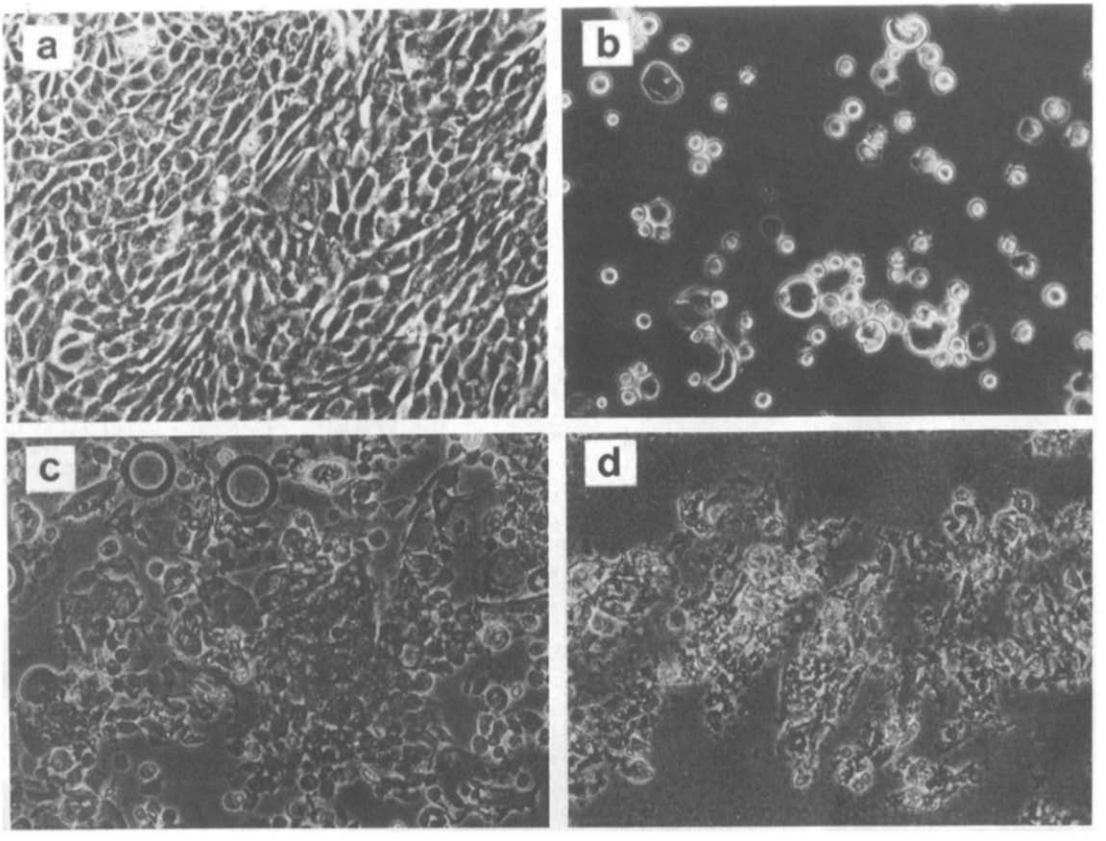

Fig. 1. Cultured human keratinocytes before and after cell disruption treatment. (*a*) Confluent keratinocyte culture before harvesting the cells for the preparation of membranes. (b) Suspension of the same cells after detachment from the culture plate. (c) Cell suspension after hypoosmotic shock (0.01 M Tris-HCl, pH 7.8), followed by 6 min homogenization in the Polytron homogenizer (a series of 20 sec homogenizations at full speed setting, followed by 1 min cooling intervals). (d) After two passages through the nitrogen cell disruption bomb.

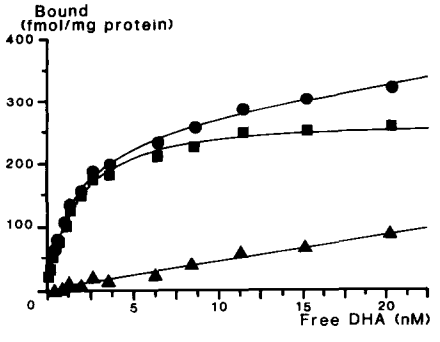

Fig. 2. Saturation binding of ^{3}H-DHA to keratinocyte membranes. A membrane suspension having a protein concentration of 2.2 mg/ml in the incubation buffer was used. Non-specific binding was determined by measuring the binding in the presence of 5 μM L-propranolol as the competitor. The binding data was computer analysed in two different ways: (1) The non-specific binding actually measured (▲–▲) was subtracted from the total binding (●–●) and the difference (■–■, specific binding) was analysed by a hyperbolic non-linear curve fitting model. (2) The total binding data were computer-analysed by adding a linear term to the hyperbolic regression model. The drawn curves are the computer fit to the data points.

Although the Scatchard and the Hill plots give a visual indication of receptor homogeneity (linearity or curvature of the Scatchard plot and the slope of the Hill plot), it is preferable to analyse the direct binding data (Fig. 2), since in this analysis the experimental data does not have to be transformed (e.g. by taking reciprocals) and the relative weight

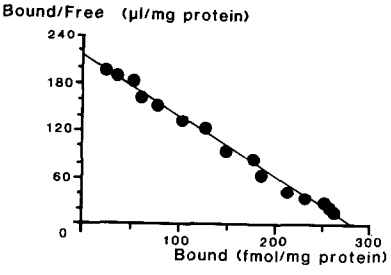

Fig. 3. Scatchard analysis of the specific binding data. Following the Scatchard transformation of the data, the points were analysed by using a linear regression algorithm. From the slope of the line, an equilibrium dissociation constant of 1.32 nM is obtained and the intercept on the X-axis yields a receptor concentration of 280 fmol/mg protein.

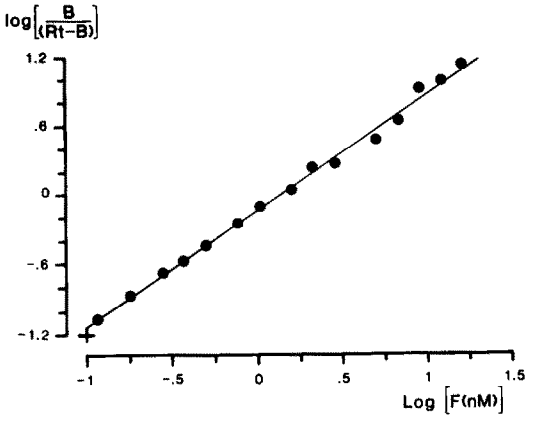

Fig. 4. Hill plot of the specific binding data. The specific binding data and the total receptor concentration calculated previously (Fig. 2) were used for the plot. The line has a slope (Hill coefficient) of 1.006 (indicating a non-interacting receptor population) and a K_d value of 1.36 nM was obtained from the calculated intercept on the X-axis. $[R_t]$ = total receptor concentration; B = bound ligand and [F] = free ligand concentration.

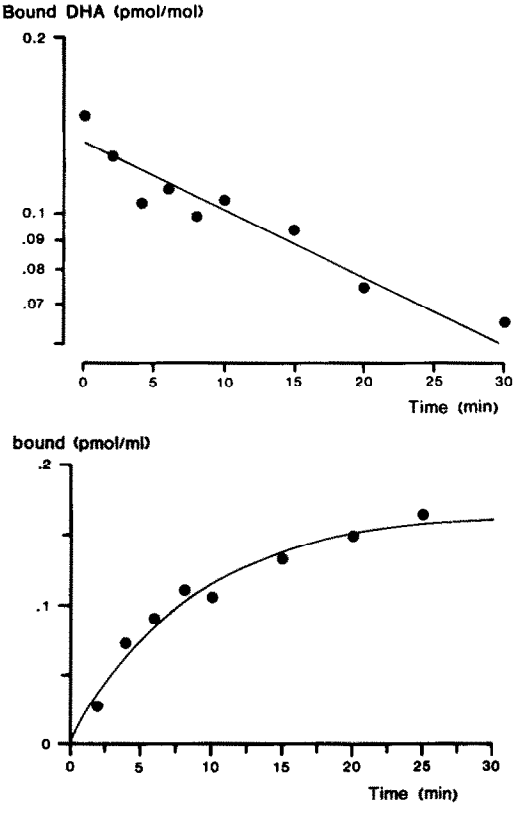

Fig. 5. (a) Dissociation kinetics of ^{3}H-DHA from keratinocyte membranes. 3 nM of ^{3}H-DHA were equilibrated with a membrane suspension (2.2 mg/ml) for 1 hr. Unlabeled L-alprenolol was added (time 0) to give a final concentration of 10 μM (3000 fold molar excess) and the amount of bound DHA was measured for the next 30 min. (b) Association kinetics of ^{3}H-DHA to keratinocyte membranes. Membrane suspension of 1.6 mg/ml was incubated with 3.4 nM ^{3}H-DHA and at the indicated times, samples were withdrawn to determine the amount of bound ligand. For the non-specific binding, another incubation mixture in the presence of 5 μM L-propranolol was run in parallel and the data points are the difference of the two curves. The association data were treated by a non-linear algorithm [21] using as a regression model the differential equation: $d[R_L]/dt = k_1 \times [R_o] \times [L_o] - (k_1 \times [L_o] + k_{-1}) \times [R_L]$; were k_1 and k_{-1} are the association and dissociation rate constants, respectively, $[R_o]$ is the total receptor concentration, $[L_o]$ is the initial concentration of the free ligand and assumed to be in high excess to $[R_o]$, and R_L represents the bound ligand. The differential equation was solved by numerical integration and both the k_1 and $[R_o]$ values could be evaluated from this experiment by using the k_{-1} value determined in the dissociation experiment. The curve drawn is the computer calculated regression line.

of the data points have not been changed by mathematical manipulations. However, such analysis of untransformed data requires the use of curve fitting algorithms and iterative procedures which have to be carefully checked against the possibility of yielding biased results due to program structure, iteration procedures, etc.

The dissociation of the receptor-ligand complex followed first order kinetics (Fig. 5(a)). The dissociation half life time determined from the semi-logarithmic plot was found to be 33 min at 25° and a dissociation rate constant of $(3.5 \pm 1) \times 10^{-4}\,\text{sec}^{-1}$ was calculated from this experiment. Since in the association of the receptor with its ligand the dissociation reaction part cannot be neglected (see equation), the dissociation rate constant found above has been used to solve the equation and to obtain the association rate constant which was calculated to be $(3.0 \pm 1.5\,\text{SD}) \times 10^5\,\text{M}^{-1}\,\text{sec}^{-1}$. The equilibrium dissociation constant (K_d) obtained from the ratio of the dissociation and association rate constants ($K_d = k_{-1}/k_1$) has a value of (1.2 ± 0.6) nM. This value is close enough to the dissociation constant obtained under equilibrium conditions to suggest that the binding under the conditions employed follow classical kinetics and can be described as a single step reaction. The receptor density found from the kinetics experiments was 285 ± 83 fmol/mg membrane protein and is in agreement with the value obtained under equilibrium conditions, lending further support to the assumption that the binding follows a simple bi-molecular reaction mechanism.

In Table 1, the affinities of several ligands with different subtype specificities are shown. The affinities reported were calculated from competition binding experiments with [^{125}I]-iodocyanopindolol and using a K_d value of 0.03 nM [21] for the iodinated ligand to calculate the dissociation constants of the unlabeled competitors. The affinity for propranolol calculated in this way (5.2 nM) differs from the value determined in saturation binding experiments (1.4 nM). Since in competition studies the affinity of the competitor is not directly determined, but is calculated later, differences in the affinity values from saturation and competition experiments are not uncommon and could be ascribed to methodological differences and accumulated errors. The relative affinities of the 3 catecholamines (isoproterenol > epinephrine > norepinephrine) are characteristic for a β_2 subtype of adrenoceptors and the K_d values of

Table 1. Binding affinities of several β-adrenergic ligands, determined from competition studies of [^{125}I]-iodocyanopindolol

Ligand	Specificity	IC_{50} (M)	K_d (M)
Alprenolol* (B)	$\beta_1 = \beta_2$		1.3×10^{-9}
Propranolol (B)	$\beta_1 = \beta_2$	1.5×10^{-8}	5.2×10^{-9}
IPS 339 (B)	$\beta_1 = \beta_2$	1.03×10^{-7}	3.4×10^{-8}
ICI 118 551 (B)	$\beta_2 > \beta_1$	1.68×10^{-8}	5.6×10^{-9}
Butoxamine (B)	$\beta_2 > \beta_1$	5.8×10^{-6}	7.8×10^{-7}
Betaxolol (B)	$\beta_1 > \beta_2$	1.07×10^{-6}	3.5×10^{-7}
Metoprolol (B)	$\beta_1 > \beta_2$	9.9×10^{-6}	1.3×10^{-6}
Practolol (B)	$\beta_1 > \beta_2$	4.95×10^{-4}	1.5×10^{-4}
Salbutamol (A)	$\beta_2 > \beta_1$	2.2×10^{-6}	2.95×10^{-7}
Isoproterenol (A)	$\beta_1 = \beta_2$	4.19×10^{-6}	5.9×10^{-7}
Epinephrine (A)	$\beta_2 > \beta_1$	3.47×10^{-5}	5.7×10^{-6}
Norepinephrine (A)	$\beta_1 > \beta_2$	5×10^{-4}	7.07×10^{-5}

A = β-adrenergic agonist.
B = β-adrenergic antagonist.
* Value determined in saturation binding studies.

the subtype specific ligands are in agreement with the published affinity values for these ligands on a β_2 population of receptors [21, 27]. In Fig. 6, the displacement of iodocyanopindolol by 3 ligands is shown: a β_1-specific antagonist (betaxolol), a β_2-specific antagonist (ICI 118 551) and IPS 339 which is a mixed antagonist, showing equal affinities to both subtypes. Each of the 3 ligands shows a monophasic displacement curve with the highest affinity exhibited by the β_2 antagonist and the lowest by the β_1 antagonist, suggesting a β_2 receptor population.

Stereoselectivity of the binding is evident since D-isoproterenol has a K_d which is at least 100 fold higher than that of the L-isomer (data not shown). The exact K_d value could not be determined due to the very high ligand concentrations required in order to obtain complete displacement of the iodinated ligand.

The density of the β-adrenergic receptors found in human keratinocyte membranes is relatively high when compared with other tissues known to be responsive to β-adrenergic stimulation such as brain cortex, cerebellum, lung, heart etc. Although the experiments presented above do not permit any conclusion concerning the state or the role of these receptors, new experimental evidence (to be published shortly) suggest that the β-adrenoceptors are functional and are coupled to an active adenylate cyclase system that can be activated by the binding of β-adrenergic agonists.

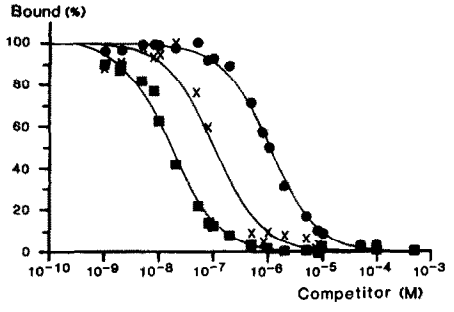

Fig. 6. Competition of [^{125}I] iodocyanopindolol by the antagonists ICI 118 551, IPS 339 and betaxolol. Membrane suspension at a concentration of 1.4 mg/ml was incubated with 65 pM of [^{125}I]-iodocyanopindolol and the binding of the iodinated ligand was measured at various concentrations of the unlabeled antagonists. The IC_{50} values for each of the antagonists were determined from regression analysis of the displacment curves. The K_d values were calculated using the following relation: $IC_{50} = K_i(1 + [L]/K_d)$ where IC_{50} is the competitor concentration giving 50% competition, $[L]$ is the concentration of the labeled ligand, K_i is the equilibrium dissociation constant of the unlabeled competitor and K_d is the equilibrium dissociation constant of the labeled ligand. A K_d value of 0.03 nM was taken for iodocyanopindolol and this value was used to calculate the K_i values for the unlabeled ligands (values in Table 1). ■–■, ICI 118 551; x–x, IPS 339; ●–●, Betaxolol.

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REFERENCES

1. J. J. Voorhees, E. A. Duell, M. Stawiski and E. R. Herrel, *Adv. cyclic nucl. Res.* **4**, 117 (1974).
2. W. D. Wicks, E. A. Duell and J. J. Voorhees, *Adv. cyclic nucl. Res.* **4**, 335 (1971).
3. W. S. Bullough and E. B. Lawrence, *Proc. R. Soc. Lond. (Biol.)* **154**, 540 (1961).
4. J. A. Powell, E. A. Duell and J. J. Voorhees, *Arch. Dermatol.* **104**, 359 (1971).
5. J. J. Voorhees and E. A. Duell, *Arch. Dermatol.* **104**, 352 (1971).
6. F. Marks and W. Rebien, *Naturwissenschaften* **59**, 41 (1972).
7. R. E. I. Cochran, J. Thomson, A. McQueen and D. G. Beevers, *Arch. Dermatol.* **112**, 1173 (1976).
8. P. M. Gaylarde, A. P. Brock and I. Sarkany, *Clin, exp. Dermatol.* **3**, 157 (1978).

9. H. Green, *Cell* **15**, 801 (1978).
10. E. K. Orenberg and D. I. Wilkinson, *Br. J. Dermatol.* **107**, Suppl. 23, 119 (1982).
11. C. Delescluse, K. Fukuyama and W. C. Epstein, *J. invest. Dermatol.* **66**, 8 (1976).
12. V. Solanki and A. W. Murray, *J. Invest. Dermatol.* **71**, 344 (1978).
13. V. Solanki and A. W. Murray, *Ind. J. exp. Biol.* **20**, 710 (1982).
14. S.-C. Liu and M. A. Karasek, *J. Invest. Dermatol.* **71**, 157 (1978).
15. J. G. Rheinwald and H. Green, *Cell* **6**, 331 (1975).
16. H. Green, O. Kehinde and J. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5665 (1979).
17. M. J. Hunter and S. L. Commerford, *Biochim. biophys. Acta* **47**, 580 (1961).
18. R. M. Dowben, T. A. Gaffey and P. A. Lynch, *FEBS Lett.* **2**, 1 (1968).
19. B. B. Wolfe, T. K. Harden and P. B. Molinoff, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1343 (1976).
20. L. T. Williams, T. B. Gore and R. J. Lefkowitz, *J. Clin. Invest.* **59**, 319 (1977).
21. G. Engel. D. Hoyer, R. Berthold and H. Wagner, *Naunyn-Schmiedeberg's archs Pharmacol.* **317**, 277 (1981).
22. A. J. Clark, *The Mode of Action of Drugs on Cells*. A. Arnold and Co., London (1933).
23. Scatchard, G., *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
24. C. M. Metzler, G. L. Elfring and A. J. McEwen, *Biometrics* **30**, 562 (1974).
25. D. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
26. H. Akaike, *Biometrika* **66**, 237 (1979).
27. T. Kendall and K. T. McCarthy, *J. Pharmacol. exp. Therap.* **222**, 600 (1982).