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CLASSIFICATION OF β-ADRENERGIC SUBTYPES IN IMMATURE RABBIT BONE MARROW ERYTHROBLASTS

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Abstract—The beta-adrenergic receptors of immature rabbit bone marrow erythroid cells (pro-erythroblasts and basophilic erythroblasts) were identified. [^{125}I]iodocyanopindolol bound to membrane preparations derived from these erythroblasts in a rapid, reversible and saturable manner. Scatchard analysis of binding data revealed a single class of binding sites (Hill coefficient of 0.954) with an apparent equilibrium dissociation constant (K_d) of 8 pM, and a density of binding sites (B_{max}) of 1.53 pM/10^6 cells, corresponding to 920 receptors per cell. The binding of [^{125}I]iodocyanopindolol was inhibited stereospecifically by concentrations of (−)-propranolol 2 orders of magnitude lower than by the (+)-isomer. Only L-isoprenaline and L-adrenaline activated the adenylate cyclase of immature rabbit erythroblasts, while L-noradrenaline, a β_1-adrenergic agonist, was inactive. The order of potency of different agonists for displacement of bound [^{125}I]iodocyanopindolol was: isoprenaline > adrenaline > noradrenaline with respective EC_{50} (concentration required for half maximal inhibition of binding) of 7.9×10^{-7} M, 1.5×10^{-5} M and 7.9×10^{-5} M. This agonist potency series did not change with differentiation of rabbit bone marrow erythroblasts. The inhibition of specific [^{125}I]iodocyanopindolol binding to immature cells by β_1- and β_2-selective drugs (noradrenaline, practolol, procaterol and butoxamine) resulted in linear Hofstee plots. The inhibition curves obtained with procaterol and butoxamine, with apparent K_d values of 3.1×10^{-9} M and 4.9×10^{-9} M, respectively, provide further evidence that the high-affinity binding sites correspond to a homogeneous β_2-receptor subtype.

In contrast to nucleated amphibian and avian erythrocytes [1–5] the non-nucleated mammalian erythrocytes were initially thought to lack hormone-sensitive adenylate cyclase activity. It was subsequently shown, however, that rat and mice [6–11] and human [8] erythrocytes contain catecholamine-responsive enzymic activity. Using adenylate cyclase measurements [11], ligand binding techniques [10, 12] and selective β_1 and β_2 adrenergic agents [13] the β-adrenergic receptors of rat reticulocytes and erythrocytes have been classified as β_2-type. A recent pharmacological study of human erythrocytes [14], however, revealed that approximately 30% of the β-receptors on these cells had β_1-specificity.

Besides differences in β-receptor classification in red blood cells there are also species differences in the stimulation of adenylate cyclase by β-adrenergic drugs. Thus while rat reticulocytes [6–11] can respond to L-isoprenaline, rabbit reticulocytes do not [15, 16]. Moreover, both the activity of adenylate cyclase and its stimulation by L-isoprenaline were observed to change with terminal erythroid cell maturation, being much higher in rat reticulocytes than erythrocytes [10–12, 17–20].

Our previous work with anaemic rabbit bone marrow erythroblasts, fractionated into cells at different stages of development, has shown that catecholamines having β_2-adrenergic specificity (L-isoprenaline and L-adrenaline) stimulated the adenylate cyclase activity, but their effect was restricted to the most immature dividing cells [15]. However, since both dividing and non-dividing cells were found to bind the selective ligand [^{125}I]iodohydroxybenzylpindolol with almost equal affinities and to have similar β-receptor densities [16], it was suggested that the loss of responsiveness to β-adrenergic stimuli was due to an uncoupling of the β-adrenergic receptors from the adenylate cyclase catalytic protein which occurs before the final cell division. Since β_1- and β_2-specific drugs were not used in our previous studies [15, 16] the identification of the β-receptor subtypes and whether their proportion changes during erythroid cell development could not be determined.

In the present study we show that both immature (dividing) and mature (non-dividing) rabbit bone marrow erythroid cells contain a single class of β-receptors of β_2-specificity.

MATERIALS AND METHODS

Animals. White male New Zealand rabbits (1.5–2.5 kg body wt) were made anaemic by five daily subcutaneous injections of 0.3 ml of neutralized 2.5% (w/v) phenylhydrazine/kg body weight. The animals were killed on day 8 after bleeding by cardiac puncture under anaesthesia.

Isolation and fractionation of erythroid cells. The bone marrow from two femurs and humerus was disaggregated in ice-cold phosphate-buffered saline by gentle pippeting. The suspension was filtered through a double layer of muslin and the cells were collected by centrifugation at 500 *g* for 10 min and washed twice in the same medium.

Bone-marrow erythroid cells were separated into fractions containing cells at different stages of development, by density gradient centrifugation on bovine serum albumin gradients as previously described

[21]. Cells found in the top 3 straw-coloured bands were the most immature cells, i.e. mainly (>70%) proerythroblasts and large basophilic erythroblasts. They were designated the dividing erythroblast fraction. Cells present in the fifth and sixth band from the top were the most mature bone marrow cells, i.e. mainly orthochromatic erythroblasts and reticulocytes and were designated the non-dividing erythroblast fraction. Cells were pelleted and washed twice with Hanks'-BSS.

Preparation of membrane ghosts. Cells (approx. 10^7) were lysed hypoosmotically with ice-cold 10 mM Tris–HCl, pH 7.5 (1 ml). Lysates were centrifuged for 15 min at 12,000 *g* and the membranes were washed twice more with successive homogenization and centrifugation at 4° in 50 mM Tris–HCl, pH 7.5 (5 ml) and were resuspended in the same buffer (0.5 ml). Protein was determined by the method of Lowry *et al.* [22], using bovine serum albumin as standard. The average membrane protein content per 10^6 cells was 20–25 μg and 10–15 μg for the dividing and non-dividing cells, respectively.

Assay of adenylate cyclase activity. Adenylate cyclase was determined by measuring the formation of cyclic AMP from ATP by the method described by Albano *et al.* [23]. The standard adenylate cyclase assay system contained (final concentrations): 2 mM ATP, 3 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 6 mM theophylline and 10 μM GTP in 50 mM Tris–HCl buffer, pH 7.4. Catecholamines were added in a 50 μl volume of final concentrations as indicated. The reaction was initiated by the addition of 50 μl of the membrane fraction (containing 15–30 μg protein), and the assay mixtures were incubated at 37° in a shaking water bath for 20 min. Reaction rates were linear for at least 30 min (data not shown). At the end of the incubation the tubes were placed in a boiling water bath for 5 min, and the assay mixtures were cooled and centrifuged at 3000 *g* for 10 min to remove insoluble material. Portions from the supernatant were taken for cyclic AMP measurement by saturation analysis as described by Albano *et al.* [24] with a cyclic AMP assay kit.

Binding of [^{125}I]iodocyanopindolol and its displacement by β-adrenergic drugs. The assay mixture for binding of [^{125}I]iodocyanopindolol consisted of 50 mM Tris–HCl (pH 7.4) and 2 mM $MgCl_2$ to which increasing concentrations of [^{125}I]iodocyanopindolol (1–100 pM) were added. The final volume of the binding assay was 1.0 ml. The membrane preparations corresponding to 5×10^6 cells were added last and the assay systems were incubated for 30 min at 37°. Samples (50 μl) were removed from the complete assay mixture to determine the total radioactivity and the remainder was poured onto Whatman GF/C glass-fibre filters, as described by Spiegel *et al.* [25]. The tubes were washed twice with 10 mM Tris–HCl buffer (pH 7.4), equilibrated at 20°, and the contents poured onto the filters. The filters were washed twice more and counted in a Packard Autogamma scintillation spectrometer. Specific binding was defined as the difference between the amount of [^{125}I]iodocyanopindolol bound in the absence (total binding) and in the presence of 10 μM L-propranolol (non-specific binding). Non-specific binding represented 10–15% of total binding.

The displacement experiments were performed as described above, except that the concentration of the radiolabelled ligand was 2.5 nM and increasing concentrations of β_1- and β_2-adrenergic drugs were added to the assay mixture before adding the membranes. GTP (10 μM final concentration) was also included in the reaction assay since this nucleotide can be removed by the membrane washings [26–28] and thereby lead to erroneous conclusions about the number of receptor subtypes present, as discussed by Minneman and Molinoff [2]).

Data presentation and analysis. For each data point the incubations were carried out in duplicate. The standard error of the determination was less than 10%. For the experiments shown in Figs 5–8 the data are presented as means of three different cell batches.

Linear regression analysis was used for drawing the Scatchard (Fig. 1), Hill (Fig. 2) and Hofstee (Fig. 8) plots and for calculating the association and dissociation rate constant of [^{125}I]iodocyanopindolol binding (Figs. 3 and 4). The K_d values of β-adrenergic drugs were calculated from the competition binding curves according to Cheng and Prusoff [29] (Table 1) and the kinetic constants of ligand binding were calculated according to Dickinson *et al.* [13].

Table 1. Dissociation constants of some β-adrenergic drugs for [^{125}I]iodocyanopindolol binding sites in immature rabbit bone marrow erythroblasts

Drug	Specificity	EC_{50} (μM)	K_d (nM)	r
(−)-Propranolol	$\beta_1 = \beta_2$ (antagonist)	0.00075	0.0017	—
(+)-Propranolol	$\beta_1 = \beta_2$ (antagonist)	0.075	0.17	—
L-Isoprenaline	$\beta_1 = \beta_2$ (agonist)	0.79	2.5	—
L-Adrenaline	$\beta_2 > \beta_1$ (agonist)	15	48.0	—
L-Noradrenaline	$\beta_1 > \beta_2$ (agonist)	79	250.0	0.980
Practolol	$\beta_1 > \beta_2$ (antagonist)	98	310.0	0.974
Procaterol	$\beta_2 > \beta_1$ (agonist)	0.97	3.1	0.993
Butoxamine	$\beta_2 > \beta_1$ (antagonist)	1.5	4.9	0.986

Dissociation constants were calculated from the data in Figs 5, 7 and 8 according to the equation: $K_d = \frac{EC_{50}}{1 + C/K_d^L}$ (as detailed in ref. 29), where C is the concentration of [^{125}I]iodocyanopindolol in the binding assay = 2.5 nM, K_d^L is the dissociation constant of [^{125}I]iodocyanopindolol = 8 pM, EC_{50} is the concentration of competing ligand which produces 50% inhibition of specific binding, r = correlation coefficient of the linear regression analysis (Fig. 8).

Chemicals. L-Isoprenaline, L-propranolol, L-adrenaline and L-noradrenaline were obtained from Sigma. [^{125}I]iodocyanopindolol (2000 Ci/mmol) and cyclic AMP assay kit were purchased from Amersham International plc. Practolol, procaterol and butoxamine were a generous gift from Dr R. Kerry and Professor M. C. Scrutton.

RESULTS

The specific binding of [^{125}I]iodocyanopindolol to membrane preparations derived from either dividing or non-dividing rabbit bone marrow erythroblasts was saturable. The Scatchard plot of the saturation curves was linear (Fig. 1) and the apparent dissociation constants were similar for both immature and mature cells (8.05 pM and 8.82 pM respectively). The number of beta-adrenergic receptors detected with saturating concentrations of [^{125}I]iodocyanopindolol was found to be 921 and 518 receptors per cell for the dividing and non-dividing erythroblasts, respectively, but the B_{max} was similar for both cell types, when expressed as fmoles of ligand bound per mg membrane protein. Thus, as suggested earlier using [^{125}I]hydroxypindolol [16], the density of receptor sites does not appear to alter during erythroid cell maturation and the observed 2-fold decrease in the number of receptors per cell is due to the decrease in size as the cells move from the dividing to the non-dividing stage.

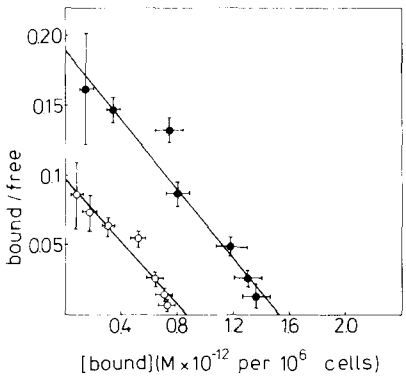

Fig. 1. Scatchard plot of the specific binding of [^{125}I]iodocyanopindolol to membranes of rabbit bone marrow dividing and non-dividing erythroblasts. Dividing (●) and non-dividing (○) erythroid cell membranes were incubated with increasing concentrations of [^{125}I]iodocyanopindolol (1–100 pM) and specific binding was determined as described in Materials and Methods. Following the Scatchard transformation of the specific binding data, the points were analysed by using a linear regression algorithm. From the slope of the lines the equilibrium dissociation constants (K_d) were obtained and the intercepts on the X-axis yield the receptor concentration (B_{max}) for both type of cells.

Dividing cells:

$$K_d = \frac{1}{\text{slope}} = \frac{\text{bound}}{\text{bound/free}} = 8.05\ \text{pM}$$

$$B_{max} = 1.53\ \text{pM}/10^6\ \text{cells (76 fmoles/mg protein)}$$

$$\text{Receptor number} = \frac{(1.53 \times 10^{-12}) \times (6.02 \times 10^{23})}{10^6 \times 10^3}$$

$$= 921\ \text{receptors/cell}$$

Non-dividing cells:

$K_d = 8.82$ pM

$B_{max} = 0.86$ pM/10^6 cells (86 fmoles/mg protein)

Receptor number = 518 receptors/cell.

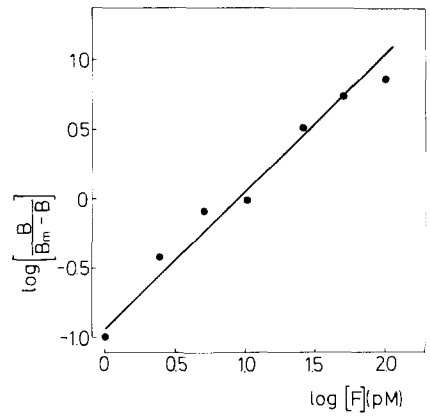

Fig. 2. Hill plot of the specific binding of [^{125}I]iodocyanopindolol. The specific binding data and the total receptor concentration calculated previously (Fig. 1) were used for the plot (determined by linear regression analysis, $r = 0.989$). The line has a slope (Hill coefficient) of 0.954. A K_d value of 8.32 pM was obtained from the calculated intercept on the Y-axis. B_m = total receptor concentration (B_{max} from the Scatchard plot), B = bound ligand, and F = free ligand.

Transformation of the data for the binding of [^{125}I]iodocyanopindolol to membrane preparations from dividing cells gave a linear Hill plot with a coefficient of unity (0.954) (Fig. 2). This indicates a uniform, non-interacting receptor population with a K_d of 8.32 pM, in complete agreement with the previous analysis.

Kinetic analysis of the binding at 37° of [^{125}I]iodocyanopindolol to rabbit immature eryth-

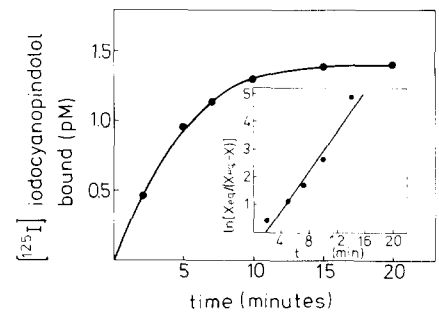

Fig. 3. Time course of [^{125}I]iodocyanopindolol binding to immature rabbit bone marrow erythroblasts. [^{125}I]iodocyanopindolol (50 pM) was incubated with immature erythroblast membranes (5×10^6 cells/ml) for the indicated times at 37° and specific binding was determined as described in Materials and Methods. *Inset*, pseudo-first order kinetic plot of [^{125}I]iodocyanopindolol binding. Data were used to determine X (amount of [^{125}I]iodocyanopindolol bound at time t) and X_{eq} (amount of [^{125}I]iodocyanopindolol bound at equilibrium = 1.4 pM). This line, determined by linear regression analysis ($r = 0.989$), has a slope, K_{ob}, of 0.348 min^{-1}, equal to the observed rate constant for the pseudo-first order reaction.

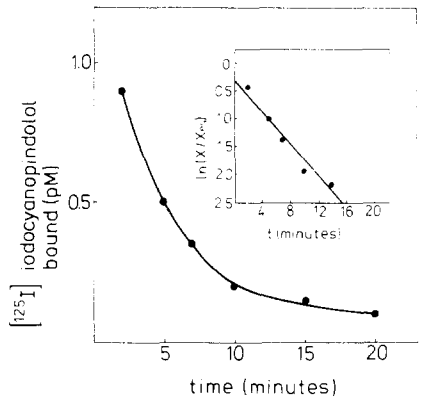

Fig. 4. Reversibility of [^{125}I]iodocyanopindolol binding to immature rabbit bone marrow erythroblasts. Erythroblast membranes (5×10^6 cells/ml) were incubated with 50 pM [^{125}I]iodocyanopindolol and after equilibrium binding was achieved (20 min at 37°) a large excess of L-isoprenaline (10 μM) was added ($t = 0$). At the indicated subsequent times, specific [^{125}I]iodocyanopindolol binding was determined. *Inset*, first order rate plot of the dissociation of the receptor–ligand complex. Data were used to construct a first order rate plot. The line, determined by linear regression analysis ($r = 0.97$), has a slope, K_2, of 0.140 min^{-1} equal to the first order rate constant. X_{eq} refers to the amount of binding present immediately prior to the addition of isoprenaline (binding at $t = 0$ was 1.4 pM) and X refers to the amount of binding present at each time t after addition of isoprenaline.

roblast membranes showed that it was rapid, with a $t_{\frac{1}{2}}$ (time for half-maximal binding) of 5 min (Fig. 3), and reversible (Fig. 4). Since the concentration of radioligand (50 pM) was much higher than the concentration of binding sites ($B_{max} = 1.53$ pM/10^6 cells), the forward (association) reaction (Fig. 3) could be considered to depend only on the concentration of binding sites (pseudo-first order reaction). This reaction can be described by the equation $\ln [X_{eq}/(X_{eq} - X)] = K_{ob}t$, where X is the amount of [^{125}I]iodocyanopindolol bound at each time (t) and X_{eq} is the amount bound at equilibrium (1.4 pM). The slope of the line in Fig. 3 (inset) is 0.348 min^{-1}, which equals the observed rate constant for the reversible pseudo-first order reaction (K_{ob}). The forward rate constant, K_1, for the second order reversible reaction between receptor and ligand can be calculated from the equation $K_1 = (K_{ob} - K_2)/[L]$ where K_2 is the rate constant for the reverse (dissociation) reaction (Fig. 4) and $[L]$ is the concentration of [^{125}I]iodocyanopindolol. K_2 was determined to be 0.140 min^{-1} and K_1 equals 4.2 nM^{-1} min^{-1}. The ratio $K_2/K_1 = 33.3$ pM is a kinetically derived estimate of the K_d for the reaction of [^{125}I]iodocyanopindolol with its binding sites. This value is comparable to the K_d (8.05 pM) determined by equilibrium studies (Scatchard and Hill plots, Figs 1 and 2).

The binding of [^{125}I]iodocyanopindolol was inhibited stereospecifically by the nonselective β-adrenergic antagonist propranolol. The concentration of (−)-propranolol producing 50% inhibition of binding was 7.5×10^{-10} M, 2 orders of magnitude lower than the inactive (+)-isomer (Fig. 5).

Figure 6 shows the concentration-effect curves for L-isoprenaline, L-adrenaline and L-noradrenaline on adenylate cyclase activity of immature rabbit erythroblasts. In keeping with our earlier results [15] only L-isoprenaline and L-adrenaline activated the adenylate cyclase of immature erythroblasts, while L-noradrenaline (a β_1-agonist) was inactive. At saturating concentrations the adenylate cyclase activity was 6 times higher in the presence of isoprenaline than in the presence of noradrenaline. Additional experiments confirmed that the lack of adenylate cyclase activation by L-noradrenaline could not be attributed to degradation of this compound during the assay. Thus when 1 mM mercaptoethanol was included in the reaction mixture to prevent oxidation the results of the adenylate cyclase activity (in pmoles cAMP formed/min/mg protein) were: control = 6.7, + 10^{-9} M L-noradrenaline = 7.5 + 10^{-4} M L-noradrenaline = 10.1. In the same experiment addition of the β_2-agonist procaterol, at 10^{-8} M or 10^{-5} M, caused a marked increase in the rate of formation of cAMP to 25.1 and 42.5 pmoles/min/mg protein, respectively.

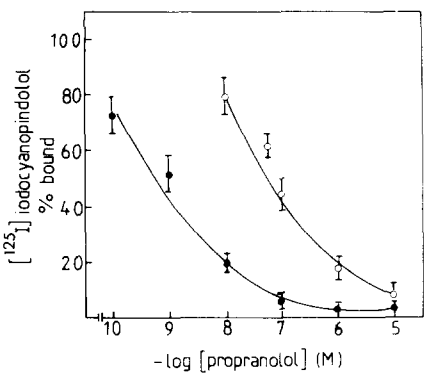

Fig. 5. Stereospecificity of inhibition of [^{125}I]iodocyanopindolol binding to immature erythroblasts by propranolol. Displacement experiments were performed as described in Materials and Methods in the presence of (−)-propranolol (●) or (+)-propranolol (○).

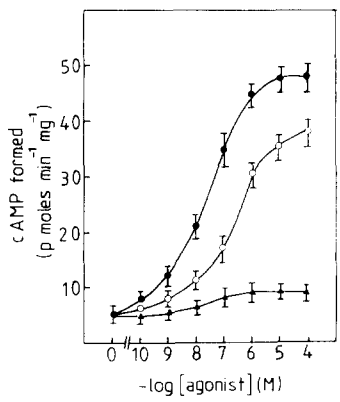

Fig. 6. Concentration–effect curves for adenylate cyclase activation by β agonists. Membranes derived from immature rabbit bone marrow erythroblasts were incubated with increasing concentrations of L-isoprenaline (●), L-adrenaline (○) or L-noradrenaline (▲) and the adenylate cyclase activity was determined as described.

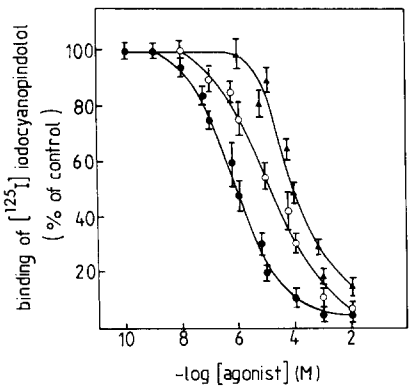

Fig. 7. Competition curves of β agonists for [^{125}I]iodocyanopindolol specific binding to plasma membrane preparations from immature erythroblasts. The standard displacement assay was performed in the presence of increasing concentrations of L-isoprenaline (●), L-adrenaline (○) or L-noradrenaline (▲).

The β-adrenergic receptors were further classified as β_2 on the basis of the order of potency of different agonists for displacement of bound [^{125}I]iodocyanopindolol which was isoprenaline > adrenaline > noradrenaline (Fig. 7). The respective EC_{50} values were 7.9×10^{-7} M, 1.5×10^{-5} M and 7.9×10^{-5} M. This agonist potency series is consistent with a β_2-adrenergic receptor subtype as defined by Lands *et al.* [30] and did not change with the differentiation of rabbit erythroblasts as identical EC_{50} values were obtained with a more mature bone marrow cell fraction, consisting of non-dividing orthochromatic cells and reticulocytes (data not shown).

To determine rigorously if only β_2-adrenergic receptors are present in membranes from early erythroid cells, we constructed competition curves with subtype selective ligands: β_1-selective agonist noradrenaline [27], β_1-selective antagonist practolol [31], β_2-selective agonist procaterol [27, 31] and β_2-selective antagonist butoxamine [32]. The inhibition of specific [^{125}I]iodocyanopindolol binding by β_1- and β_2-selective drugs resulted in linear Hofstee plots, within the limits of error implicit in this type of graphic analysis [28]. Figure 8 indicates that there is only a single class of binding sites with K_d values of 2.5×10^{-7} M and 3.1×10^{-7} M for noradrenaline and practolol and 3.1×10^{-9} M and 4.9×10^{-9} M for procaterol and butoxamine, respectively. The inhibition curves obtained suggest that the high-affinity sites correspond to β_2-receptor subtype.

The EC_{50} and K_d values calculated from Figs 5, 7 and 8 for the various β-adrenergic drugs used in competition of [^{125}I]iodocyanopindolol binding are summarized in Table 1.

DISCUSSION

The data presented here confirm and extend our previous studies [15, 16] and represent the first identification of the β-adrenergic receptor subtypes on immature rabbit erythroblasts. The displacement

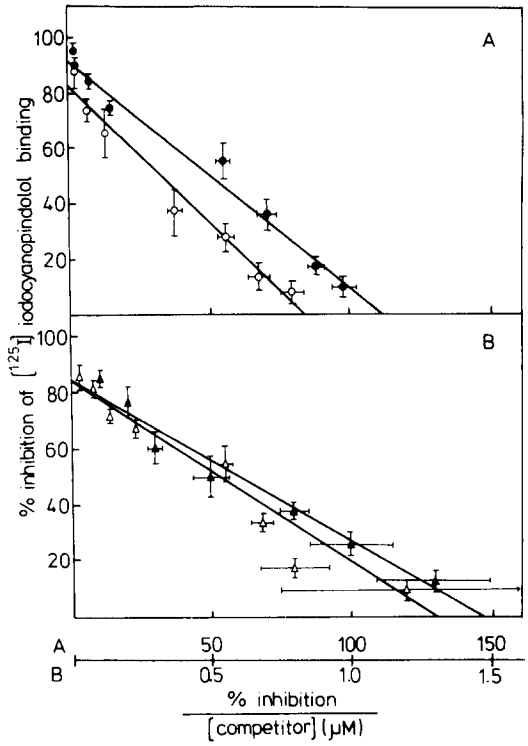

Fig. 8. Hofstee plots for the inhibition of specific [^{125}I]iodocyanopindolol binding by β_1 and β_2 agonists and antagonists. The inhibition of specific [^{125}I]iodocyanopindolol binding by selective agonists and antagonists was performed as described in Methods. (A) (β_2-specific drugs) ●, procaterol (agonist); ○, butoxamine (antagonist). (B) (β_1-specific drugs) ▲, noradrenaline (agonist); △, practolol (antagonist). The lines were drawn by linear regression analysis with correlation coefficients (r) of 0.993 (●), 0.986 (○), 0.980 (▲) and 0.974 (△).

curves of β_1- and β_2-selective adrenergic drugs in competition with [^{125}I]iodocyanopindolol binding to rabbit bone marrow erythroid cells are indicative of a homogeneous β_2-receptor population and this β_2-specificity was maintained throughout all states of differentiation. Thus our results are in agreement with the β_2-receptor subtype found in circulating rat reticulocytes and erythrocytes [10–13] and differ from the reported presence of approximately 30% β_1-receptors on human red blood cells [14].

The significance of the occurrence of the β_1- and β_2-receptor subtypes is not clear, although it has been hypothesized that it is somehow related to the ratio of circulating noradrenaline to adrenaline in various species [33]. However, a clear correlation cannot always be found [14].

The adenylate cyclase-coupled β-adrenergic receptor complex has been shown to be present in normal haematopoietic [34–36] as well as in Friend [37, 38] and Rauscher erythroleukaemia cells [39]. Although little is known about the developmental significance of the β-adrenergic adenylate cyclase system in these cells the presence of a coupled β-adrenergic receptor–adenylate cyclase complex suggests that β-adrenergic stimuli may be involved in the development and maturation of erythroid cells *in vivo*. Such stim-

uli have been shown to affect the movement of pluripotent haematopoietic stem cells from the G-0 to the G-1 phase of the cell cycle [40]. An increase of the erythropoietin-induced erythroid cell development has been demonstrated in canine bone marrow in the presence of β-adrenergic agonists [41]. The effect was mediated by β_2-adrenergic receptors since propranolol and butoxamine both inhibited the response. Recently Sytkowski and Kessler [42] have demonstrated that erythropoietin-induced differentiation of Rauscher murine erythroleukaemia cells results in a specific increase in β-adrenergic receptor density on the cell membrane. The significance of this was not clear, however, since the new erythropoietin-induced receptors were apparently not coupled to the adenylate cyclase catalytic protein, whereas the β-receptors present on uninduced and maintained on induced cells were functionally linked to the cyclase enzyme [42].

A loss of catecholamine sensitivity during the maturation of rat reticulocytes to erythrocytes has been reported [9–12, 17–20]. We found [15, 16] that uncoupling of the β-adrenergic receptors from adenylate cyclase in rabbit erythroid cells occurs much earlier in differentiation after the final cell division in the bone marrow. Uncoupling was accompanied by a decrease in the number of β-receptors both in rat reticulocytes [10–12, 17–20] and rabbit erythroblasts [16], but this was more apparent than real when the decrease in cell surface area was taken into account.

The persistence of β-receptors during differentiation of erythroid cells is different from other cell systems, wherein the loss of catecholamine responsiveness is closely associated with a concomitant loss of demonstrable β-receptors [43, 44]. Thus in rabbit erythroid cell differentiation there appears to be no difference between β-receptor subtype and β-receptor density whether the β-receptor is coupled to the adenylate cyclase catalytic subunit or not.

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