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High level of α2-adrenoceptor in rat foetal liver and placenta is due to α2B-subtype expression in haematopoietic cells of the erythrocyte lineage

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1 Rat foetal liver contains large amounts of α2-adrenoceptors. The present work aimed to identify the receptor subtype and the cell type accounting for high expression and to clarify the mechanisms responsible for the sharp decrease in hepatic receptivity occurring during the late stage of foetal development.

2 Binding experiments indicated that the density of α2-adrenoceptors in the foetal liver (embryonic day 18; 615 ± 155 fmol mg^{-1} of protein) is 18 fold higher than in newborn or adult (35.2 ± 4.3 fmol mg^{-1}). A high amount of receptor is also found in the placenta (443 ± 53 fmol mg^{-1}). In both tissues, the rank order of antagonists to inhibit radioligand binding matched the pharmacological profile of the α2B-adrenoceptor and exclusively RNG transcripts were detected by RNase protection assays.

3 Isolation of cell fractions from foetal liver showed that α2B-adrenoceptor is primarily expressed by haematopoietic cells. Consistent with this view, the receptor is found to be abundant in foetal blood, carried by reticulocytes. The expression in blood gradually declines to zero at 3 weeks of age and it is not recovered following induction of reticulocytosis in adults.

4 In foetal reticulocytes, a low proportion of the receptor population is coupled to G-protein. The α2-agonist UK14304 has a marginal effect on cyclic AMP level but significantly increases arachidonic acid release. The function of the receptor remains to be elucidated. However, together with observations on α2B-knockout mice, the current finding strongly suggests a role for α2B-adrenoceptor during foetal haematopoiesis in rodents.

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Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; MK912, 2s, 12bs-1′,3′ dimethyspiro(1,3,4,5′,6,6′,7,12b-octahydro-2H-benzo[b]furo [2,3-a] quinazoline)-2,4′-pyrimidin-2′one; PBS, phosphate buffered saline; RX821002, 2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline; UK14304, 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline

Introduction

The α2-adrenoceptor family comprises three subtypes (namely, α2A, α2B and α2C) that mediate their effects through activation of Gi/Go proteins (reviewed in Bylund *et al*., 1994). Although all receptors display similar affinity for their natural ligands, adrenaline and noradrenaline, they exhibit marked differences in pharmacological and biochemical properties. Thus, unlike the other two subtypes, the α2B-adrenoceptor is not glycosylated and it exhibits a 10 fold higher affinity for prazosin than for oxymetazoline. Transfection experiments carried out in differentiated cells also suggest a peculiarity in the metabolism and in the addressing of this subtype to the apical and basolateral membrane compartments (Wozniak & Limbird, 1996).

In the rat, the α2B-adrenoceptor is encoded by the RNG gene (Zeng *et al*., 1990). As shown by RNase protection assays (Blaxall *et al*., 1994; Handy *et al*., 1993) or by *in situ* hybridization studies (Meister *et al*., 1994), RNG is, in the adult rat, primarily expressed in kidney. Much less is found in the liver or lung, whereas only trace amounts are detected in the brain, skeletal muscle and heart. In agreement with this pattern of RNA distribution, radioligand binding experiments showed that α2B-adrenoceptor is the major subtype expressed in the rat kidney (Uhlen & Wikberg, 1991). Substantial quantities of RNG transcript and of the corresponding receptor are also found in neonatal rat lung (Bylund *et al*., 1988; Handy *et al*., 1993). In this tissue, which is presently considered as the prototype for α2B-adrenoceptor (Bylund *et al*., 1994), it is remarkable that receptor expression decreases rapidly so that it becomes undetectable at 5 weeks of age (Latifpour & Bylund, 1983). The mechanisms underlying this disappearance and the role of the receptor in the newborn are unknown.

Until very recently, the precise functions of the α2B-adrenoceptor remained rather elusive. Immunohistological

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studies on the rat kidney demonstrated that the α2B-adrenoceptor is predominantly located in the basolateral membrane of the proximal tubule (Huang *et al*., 1996) where it accelerates sodium reabsorption through the stimulation of Na^+/H^+ exchanger (NHE3). On the other hand, studies of the effect of clonidine on urine flow rate suggested that the renal α2B-subtype is responsible for increased free water clearance (Intengan & Smyth, 1996). Valuable insight into the specific functions of the α2B-subtype has been provided more recently through the development of genetically engineered mice lacking individual α2-adrenoceptor subtypes (Link *et al*., 1996). The measurement of haemodynamic parameters, following administration of dexmedetomidine, indicated that the initial transient hypertension phase was lost in α2B-knockout mice. This subtype is thus responsible for the effect of α2-agonists on vascular smooth muscle. The α2B-deficient mice were also found to be refractory to the hypertensive effect of salt-loading (Makaritsis *et al*., 1999), but the origin of this resistance is unclear. Yet, the consequences of the deletion of the α2B-adrenoceptor on other biological functions were not investigated.

At this point, it is noteworthy that α2B-knockout mice do not breed well (Makaritsis *et al*., 1999). In addition, contrary to other subtypes, heterozygote intercrosses produced 50% fewer homozygotes than predicted by Mendelian ratios (Link *et al*., 1996), suggesting a putative role for α2B-subtype during development.

A former study of the ontogeny of hepatic adrenoceptors showed that the liver from rat embryos contains large amounts of α2-adrenoceptors (McMillian *et al*., 1983). The aims of the present work were to identify the receptor subtype and the cell population accounting for its high expression. Since the liver is the principal site of haematopoiesis during foetal life and because receptor expression rapidly falls by birth, it was hypothesized that the receptor may be correlated with this function. Our results indicate that the α2-adrenoceptor of rat foetal liver is of the α2B-subtype and that it is borne by cells of erythroblastic lineage. The current observation strongly suggests a heretofore unappreciated role for this receptor subtype during foetal haematopoiesis.

Methods

Sources of tissues

Wistar rats and B6D2/F1 mice were respectively purchased from Harlan France (Malcourlet, France) and Charles River/Iffa-Credo (L'Arbresle, France). Timed pregnant females at 18 days post-coitus were anaesthetized by a single intraperitoneal injection of pentobarbitone (50 mg kg^{-1}) and their embryos were harvested by Caesarean section. Unless otherwise indicated, foetal tissues were rapidly dissected, washed in ice-cold PBS (phosphate buffered saline), frozen in liquid nitrogen and stored at $-80°C$ until analysis. Neonatal and adult tissues were collected under identical conditions. The mixed-sex litters were used for foetuses and newborns. All experiments were conducted in accordance with guiding principles for the care and use of animals. Human umbilical cord blood was obtained from normal full-term pregnancies and collected according to institutional guidelines.

Induction of reticulocytosis

Reticulocytosis was induced by serial phlebotomies combined with erythropoietin treatment. Male rats (mean weight 250 g) were anaesthetized by inhalation of isofluorane/O_2 (5 : 95). They were bled by heart puncture (20% of blood volume, every third day for 6 days) and received intravenous saline solution (caudal vein) for volumetric compensation. Recombinant human erythropoietin (rHuEpo) was administered daily by intraperitoneal injection (200 iu kg^{-1}).

Haematology and fractionation of blood cells

Blood was collected on EDTA and parameters (haemoglobin, haematocrit, cell counts, reticulocyte index) were determined using an automated system (Sysmex Europe, Villepinte, France). The separation of blood cells was achieved by centrifugation on an iso-osmotic Percoll gradient (Amersham Pharmacia Biotech, U.K.). Briefly, blood samples were diluted (1 : 1) in PBS, layered at the top of a discontinuous gradient (1.065, 1.09 and 1.1 g ml^{-1}) and centrifuged at $800 \times g$ for 10 min at 25°C. The layers corresponding to the different cell types (thrombocytes, lymphocytes, granulocytes and erythrocytes) were collected, washed in HEPES-buffered Dulbecco's Modified Eagle's Medium (DMEM) (10 mM HEPES, pH 7.4) before use for further experiments.

Separation of foetal liver cells

Foetal liver cells were separated according to a method described previously (Moncany & Plas, 1980). Liver was cut into small pieces and cells were dissociated by incubation in HEPES-buffered saline (mM): HEPES 125, NaCl 67, KCl 6.7, $CaCl_2$ 5, glucose 100, pH 7.6, containing 0.2% collagenase and 100 μg ml^{-1} DNase. After 30 min at 37°C, the preparation was filtered through a layer of gauze and cells were collected by gentle centrifugation ($50 \times g$, 10 min). The pellet was taken up in DMEM supplemented with 10% foetal calf serum and cells were seeded on collagen-coated dishes. After 12 h of incubation at 37°C under a 5% CO_2 atmosphere, hepatocytes and fibrobasts adhered firmly to the substratum while haematopoietic cells remained in suspension.

Membrane preparation and receptor quantification

Tissues or isolated cells were homogenized in TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5) and centrifuged at $27{,}000 \times g$ for 10 min at 4°C. The particulate fraction was washed three times in TM buffer (50 mM Tris-HCl, 0.5 mM MgCl2, pH 7.5) and the final crude membrane pellet was used for α2-adrenoceptor quantification using [^{3}H]-RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline), [^{3}H]-MK912 (2s,12bs-1′,3′ dimethyspiro (1,3,4,5′,6,6′,7,12b-octahydro-2H-benzo[b]furo [2,3-a] quinazoline)-2,4′-pyrimidin-2′one) or [^{3}H]-clonidine as radioligand. Binding experiments were carried out as previously described (Devedjian *et al*., 1994). The membrane pellet was resuspended in the appropriate volume of TM buffer. Total binding was measured by incubating 100 μl of membrane with the radioligand in a total volume of 400 μl of TM buffer. After 45 min at 25°C, bound radioactivity was separated from free by filtration through GF/C Whatman filters using a Millipore manifold

sampling unit. Filters were rapidly washed with ice-cold TM buffer and membrane-bound radioactivity was determined by liquid spectrometry. Specific binding was defined as the difference between total and non-specific binding measured in the presence of 1×10^{-5} M phentolamine. For saturation experiments the final concentration of radioligand ranged from 0.01 to 4 nM for [^{3}H]-MK912 and from 0.5 to 16 nM for [^{3}H]-RX821002 or [^{3}H]-clonidine. For inhibition studies, the indicated concentrations of competitor were added to the incubation mixture before addition of the membrane suspension. β-adrenoceptors were quantified in the same way, but using [^{125}I]-cyanopindolol (final concentration from 0.01 to 0.45 nM) and 1×10^{-6} M bupranolol to determine non-specific binding.

Synthesis of subtype-specific probes and RNase protection assays (RPA)

The antisense riboprobes for detection of receptor transcripts were obtained by subcloning regions of RG10, RG20 and RNG genes into pBluescriptll KS+ (pKS+, Stratagene, La Jolla, CA, U.S.A.). The probes for α2A- and α2C-subtypes consisted of *Nar*I-*Nar*I fragments corresponding respectively to nucleotides 587–879 of the RG20 sequence and to nucleotides 824–1067 of the RG10 sequence. That for the α2B-subtype was the *Pst*I-*Pst*I fragment corresponding to nucleotides 628–897 of the RNG sequence. The probe for GAPDH corresponded to nucleotides 19–196 of the cDNA of rat GAPDH. Orientation of the constructs was determined by sequencing. The plasmids were cut with the appropriate restriction enzyme and the antisense labelled RNAs were synthesized in the presence of [α^{32}P]-UTP using the T3 or T7 RNA polymerase (Promega, Madison, WI, U.S.A.). Cellular RNAs were isolated using the guanidinium isothiocyanate/phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). The integrity of the preparations was assessed by agarose gel electrophoresis and their concentrations were determined by UV spectrophotometry. RPA were performed on 50 μg of total RNA as previously described (Devedjian *et al*., 1991).

Measurement of cyclic AMP production and of PLA2 activity

For the measurement of cyclic AMP, cells were incubated in a 200 μl final volume of HEPES-buffered DMEM containing the drug to be tested. After a 20 min period at 37°C, the reaction was terminated by the addition of 1.8 ml of formic acid/methanol (5:95). Aliquots of the cleared supernatant were dried, resuspended in assay buffer (0.1 M Tris, 1 mM EDTA, 0.02% sodium azide) and their cyclic AMP content determined by RIA. PLA2 activity was estimated by measurement of arachidonic acid release. Cells in HEPES-buffered DMEM containing 20 mM nordihydroguaiaretic acid were labelled with 1 μCi ml^{-1} [^{3}H]-arachidonic acid. After a 1 h-period of loading at 37°C, cells were extensively washed in the same medium supplemented with 0.2% fatty acid-free BSA and treated or not with the α2-agonist UK14304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline). At timed intervals, aliquots of culture medium were collected, centrifuged and the radioactivity in the supernatant was counted by liquid scintillation spectrometry.

Drugs and chemicals

[^{3}H]-MK912 (79 Ci mmol^{-1}), [^{3}H]-clonidine (67 Ci mmol^{-1}) and [^{125}I]-cyanopindolol (2000 Ci mmol^{-1}) were from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]-RX821002 (59 Ci mmol^{-1}) was from Amersham (Amersham, U.K.) and [α^{32}P]-UTP from ICN (Costa Mesa, CA, U.S.A.). Phentolamine and clonidine were donated by Ciba-Geigy (Basle, Switzerland) and Boehringer-Ingelheim (Ingelheim, Germany). UK14304 and RX821002 were gifts from Pfizer (Sandwich, U.K.) and Reckitt and Colman Laboratories (Kingston-upon-Hull, U.K.). BRL41992 and BRL44408 were from Beecham Pharmaceuticals (Epsom, U.K.) and imiloxan from Syntex Laboratories Inc. (Palo Alto, CA, U.S.A.). RNase A, RNase T1 and proteinase K were purchased from Boehringer Mannheim (Meylan, France). Yohimbine, (−)adrenaline, oxymetazoline, chlorpromazine, WB4101, corynanthine, pertussis toxin, GppNHp, forskolin, and all other chemicals were from Sigma (St Louis, MO, U.S.A.). Foetal calf serum was purchased from Gibco-BRL (Cergy Pontoise, France). Radioimmunoassay kits for cyclic AMP determination were from Immunotech (Luminy, France).

Analysis of data

The protein concentration was determined using the Coomassie blue method (Bradford, 1976). Binding data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.). All data are expressed as means ± s.e.mean. Statistical analysis was performed by Student's *t*-test and *P* values less than 0.01 are indicated with asterisks (**).

Results

Foetal liver and placenta express high levels of α2B-adrenoceptor

A previous study of the ontogeny of adrenoceptors in the rat liver showed important changes in the density of [^{3}H]-rauwolscine binding sites during foetal life (McMillian *et al*., 1983). So far, rather little attention has been given to this observation, so that this phenomenon remained unexplored. The first aim of the present work was therefore to confirm previous findings and to define the receptor subtype responsible for the marked fall in α2-adrenergic receptivity occurring during the last days of gestation. Binding of [^{3}H]-MK912 was thus examined on crude membranes prepared from different tissues including foetal and adult liver and placenta. In addition, neonatal rat lung was taken as reference (Figure 1, left panel). Of the four tissues, foetal liver exhibited the highest density of binding sites (615 ± 155 fmol mg^{-1} of protein, $n=8$). Such a B_{max} value is 18 fold that in adult liver and 3 fold that in neonatal lung. Unexpectedly, a considerable number of [^{3}H]-MK912 binding sites were also found in placenta (443 ± 85 fmol mg^{-1} of protein, $n=4$), a tissue which has never been assayed before for α2-adrenoceptor expression. Identical results were obtained when [^{3}H]-RX821002 was used as a radioligand. As reported in Table 1, the K_D values of the two radioligands in foetal liver and placenta were nearly identical to that in

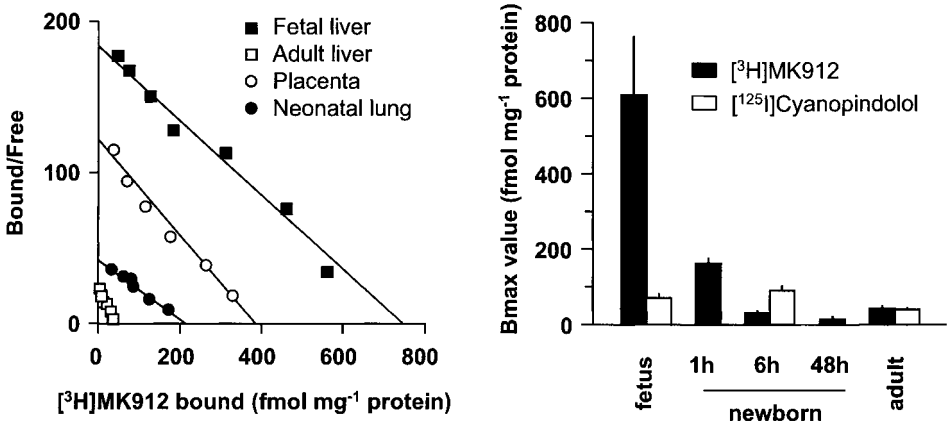

Figure 1 Expression of α2-adrenoceptors in the liver, placenta and lung. Left panel: Scatchard plots of [^{3}H]-MK912 binding to membrane prepared from foetal liver, adult liver, placenta and neonatal lung. The presented data are from a typical experiment and each point represents the mean of duplicates. Right panel: Evolution of hepatic α2- and β-adrenoceptors as a function of age. The number of [^{3}H]-MK912 and [^{125}I]-cyanopindolol binding sites was measured in membranes from liver of foetuses, newborns at 1 h, 6 h or 2 days of age and adult rats. Results are means±s.e.mean from five independent experiments.

Table 1 Binding parameters of [^{3}H]-MK912 and [^{3}H]-RX821002

	[^{3}H]-MK912		*[^{3}H]-RX821002*	
Tissue	B_{max}	K_D	B_{max}	K_D
Foetal liver	615±155 (n=8)	1.78±0.24	559±89 (n=5)	6.46±1.02
Adult liver	35.2±4.3 (n=8)	1.37±0.11	41±9 (n=4)	8.6±1.7
Placenta	443±53 (n=4)	1.21±0.3	382±35 (n=4)	4.27±0.9
Neonatal lung	243±22 (n=3)	2.11±0.51	ND	ND

Membranes prepared from foetal and adult liver, placenta and neonatal lung were incubated with increasing concentrations of [^{3}H]-MK912 or [^{3}H]-RX821002 and specific binding was determined as described in the Methods section. Computer-assisted analysis of the data indicated that the two radioligands labelled a single class of binding sites. The maximum number of binding sites (B_{max}) and the dissociation constant value (K_D) were calculated by non-linear regression analysis according to a one-component model. B_{max} and K_D are respectively expressed in fmoles mg^{-1} of protein and in nM. Reported values are means±s.e.mean from n (in parentheses) determinations (ND: not determined).

neonatal lung, suggesting that the three tissues express the same receptor subtype. At this point, it is also noteworthy that very marked levels of receptor expression were found when mouse foetal liver and placenta were assayed. The kinetics of decrease were investigated in livers of rats at different ages (Figure 1, right panel). In agreement with previous data (McMillian *et al*., 1983), the fall in α2-adrenoceptor levels occurred very rapidly during the last days of gestation. Indeed, the number of [^{3}H]-MK912 binding sites at birth was approximately 25% of that at day 18 of embryonic development. Moreover, receptor density in liver from 6 h-old newborns was identical to that in adult. During the same period, the number of binding sites for [^{125}I]-cyanopindolol did not change significantly, indicating that β-adrenoceptors were not affected by this process. The subtype of α2-adrenoceptor present in foetal liver was assessed pharmacologically by comparing the ability of subtype-selective drugs to prevent radioligand binding. As shown in Figure 2, prazosin inhibited [^{3}H]-MK912 binding with a higher potency than oxymetazoline. Furthermore, the EC_{50} value for the α2B-selective compound BRL41992 (Young *et al*., 1989) was two orders of magnitude lower than for BRL44408. The results from competition studies performed with a larger series of antagonists are summarized in Table 2. All compounds inhibited [^{3}H]-MK912 binding according to a

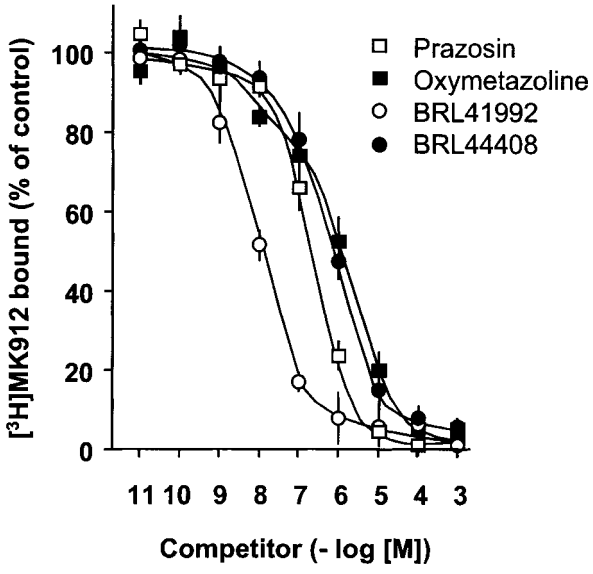

Figure 2 Inhibition of [^{3}H]-MK912 binding by subtype-selective compounds. Binding studies were performed as described in the Methods section. Concentration of [^{3}H]-MK912 was 3 nM. Prazosin, oxymetazoline, BRL41992 and BRL44408 were tested as competitor. Values of EC_{50} were calculated using GraphPad Prism. The values of K_i for prazosin, BRL41992 and BRL44408 are given in Table 2.

Table 2 Inhibition of [^{3}H]-MK912 binding by adrenergic compounds

Compound	K_i (nM)	*Compound*	K_i (nM)
BRL41992	5.8±2.5	Imiloxan	192±15
Yohimbine	24.6±9	Prazosin	205±25
WB4101	41.9±16	BRL44408	464±40
Chlorpromazine	45.3±5	Corynanthine	627±30

Membranes from foetal liver were incubated in the presence of 3 nM [^{3}H]-MK912 and increasing concentrations of competitor as described in the Methods section. Data were analysed according to a one-site model. Reported K_i values are means±s.e.mean from three determinations.

monophasic model and with a rank order of potency (BRL41992 > yohimbine > WB4101 = chlorpromazine > imiloxan = prazosin > BRL44408 ≥ corynanthine) which is consistent with foetal liver receptor being of the α2B-subtype. Identical results were obtained when placenta membranes were used. This conclusion was further confirmed by RPA. No protected band was revealed when RNAs prepared from foetal liver or placenta were hybridized with the RG10 or RG20 riboprobes, which are specific for rat α2C- and α2A-adrenoceptors respectively (not shown). In sharp contrast, a strong signal was obtained when RNG probe was used (Figure 3). The results from RPA were moreover consistent with the observed changes in hepatic receptivity, as the amount of RNG transcript in the neonatal liver was considerably less than that in foetus and fairly similar to that in adult.

α2B-adrenoceptor is carried by haematopoietic cells of the erythroblastic lineage

The rat foetal liver is an organ of transient but intense haematopoietic activity during mid gestation, with erythropoiesis being predominant between days 11 and 17. In order to examine the possibility that high expression of α2B-adrenoceptor is related to this unique function, haematopoietic cells were isolated using a culture technique allowing their separation from hepatocytes (Moncany & Plas, 1980). Measurement of [^{3}H]-MK912 binding, on membranes prepared from attached and floating cells (see Methods section), clearly indicated that the receptor was not carried by hepatocytes or fibroblasts, but by cells of haematopoietic origin (not shown). Consistent with this view, a high level of receptor was also found in blood collected from perfused placenta. Expression was therefore examined on blood collected from foetus at late stage of development (day 20), as well as from neonate, young and adult rat. As shown on the left panel of Figure 4, crude membranes prepared from foetal and neonatal blood exhibited an extraordinarily high density of [^{3}H]-MK912 binding sites (~2 pmol mg^{-1} of protein). The expression started to decline significantly high after day 3 of life. Expression continued until day 14, but was no longer detectable after day 21. Again, the temporal pattern of receptor expression was consistent with the decline and disappearance of its transcript. Indeed, substantial amounts of RNG mRNA were found in blood from foetus and newborn, but not from young (2 weeks of age) or adult. On the other hand, the level of receptor correlated

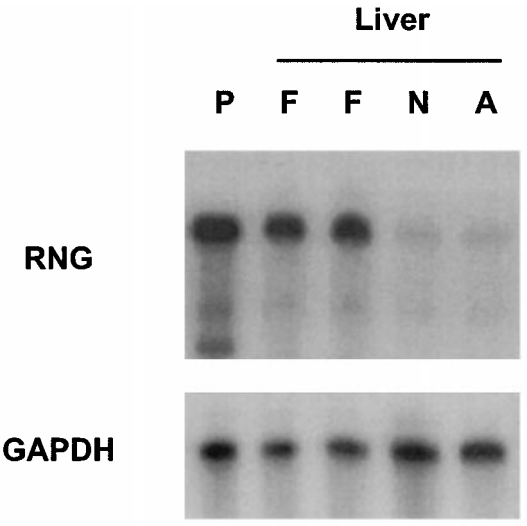

Figure 3 Identification of α2-adrenoceptor transcripts. RNAs from placenta and liver were hybridized with ^{32}P-labelled riboprobe for RNG (upper panel) or GAPDH (lower panel). Samples were digested with a mixture of RNases A and T1. The resistant hybrids were electrophoresed and gels autoradiographed. Lanes P, F, N and A correspond respectively to RNA extracted from placenta, foetal liver, neonate liver and adult liver.

remarkably well with the percentage of reticulocytes (100% at day 1, 29% at day 7, 14% at day 14, 13% at day 21 and 5% in adult), suggesting that cells of erythroblastic origin are responsible for receptor expression. Blood cells were therefore fractionated in order to identify more precisely the cell type responsible for expression of α2B-adrenoceptor. Of the four fractions (thrombocytes, monocytes, granulocytes, erythrocytes) that were separated by sedimentation on a discontinuous Percoll gradient, [^{3}H]-MK912 binding was almost totally recovered in red cells, confirming that the receptor is expressed by reticulocytes (Figure 4, right panel). Two questions were addressed next. Firstly, is it possible to induce receptor reappearance in the adult rat? Secondly, are the results found in rat also valid in man? The first point was examined by induction of reticulocytosis in adult rats. Repeated bleeding combined with erythropoietin administration resulted in a 3 fold increase of the percentage of reticulocytes (6±1% of the red cell population in control versus 19±2% in treated rats). However, this rise was not accompanied by restoration of the receptivity (not shown), suggesting that only foetal reticulocytes express the receptor. On the other hand, the situation in man was explored by studying the binding capacity of membranes prepared from umbilical cord blood collected at full-term pregnancy. Experiments with [^{3}H]-MK912 showed the presence of only 76±12 fmol of sites per mg of protein (K_D = 1.26±0.36, n = 5). According to inhibition by prazosin and oxymetazoline, this site is of α2A-subtype and it likely corresponds to platelet receptor.

Receptor functionality

The last series of experiments were designed to estimate receptor functionality and to examine the putative pathways of signal transduction. Coupling of the receptor to

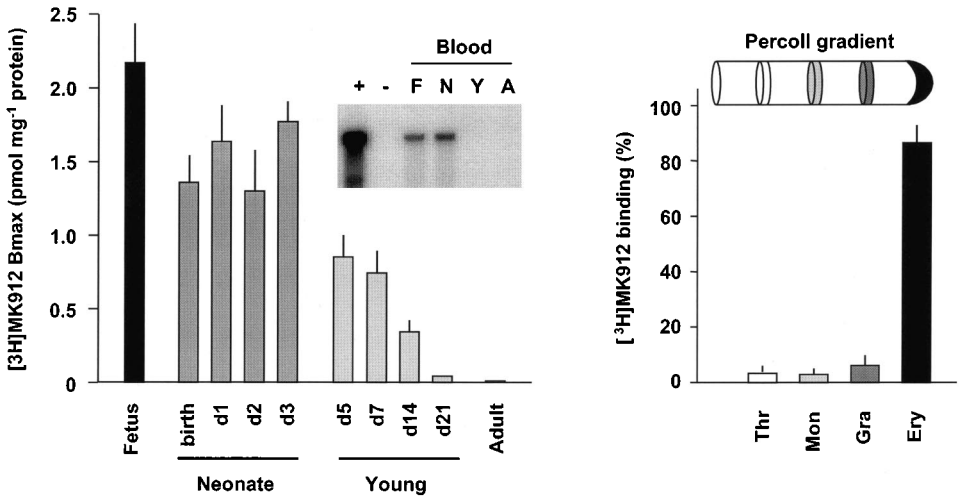

Figure 4 Receptor expression in blood. Left panel: [^{3}H]-MK912 binding was examined on membrane prepared from whole blood collected from foetus (day 20 of development), newborn (at birth, day 1, 2 and 3), young (at day 5, 7, 14 and 21), or adult rats. Inset: the amounts of RNG transcript were measured by RPA as detailed in the legend of Figure 3. Lanes + and − correspond to RNA from COS-7 cells transfected or not with the RNG gene. Lanes F, N, Y and A correspond to RNA extracted from blood collected from foetus, newborn (day 3), young (day 14) and adult. Right panel: Blood collected from foetus at day 20 of development was centrifuged on discontinuous Percoll gradient as detailed in the Methods section. Four fractions were separated, corresponding from top to bottom to thrombocytes (Thr), monocytes (Mon), granulocytes (Gra) and erythrocytes (Ery). α2-adrenoceptor number was estimated by analysis of [^{3}H]-MK912 saturation isotherms. Binding is expressed as per cent of that in whole blood. Reported values are mean±s.e.mean from three independent experiments.

G proteins was first evaluated by studying the inhibition of [^{3}H]-MK912 binding by the physiological agonist, (−)adrenaline, in the presence or absence of GppNHp plus Na^+ (Figure 5). Under control conditions, data were better fitted according to a two-component model. Analysis of three independent experiments showed that the fraction of receptor under high-affinity state for the agonist represented 13.5±3.6% of the whole receptor population and that the K_i value of (−)adrenaline for high- and low-affinity conformation was respectively 6.2±2.7 and 1865±754 nM. Addition of GppNHp plus Na^+ resulted in complete abolition of the fraction of receptor with high affinity for the agonist. Results from competition experiments with (−)adrenaline were reinforced by direct study of [^{3}H]-clonidine binding. Indeed, the agonist labelled with high affinity (K_D=1.5 nM) a single class of sites, but its B_{max} value was considerably lower than with [^{3}H]-MK912 (the ratio [^{3}H]-clonidine B_{max}/[^{3}H]-MK912 B_{max} was 0.18 in placenta and 0.10 in blood from newborn). Thus a rather low fraction of the receptor population is coupled to G-protein. However, given that foetal reticulocytes express enormous quantities of receptor, this fraction represents a number of coupled receptors that is expected to be capable of triggering a biological effect. The effect of α2-agonist was assayed on cyclic AMP production and arachidonic acid release (Figure 6). As expected, exposure of foetal reticulocytes to forskolin evoked a 22 fold increase in cyclic AMP level. A trend toward a diminution of cyclic AMP accumulation was repeatedly observed when UK14304 was added. However, the decrease was marginal and not statistically significant. By contrast, a slight but significant increase in arachidonic acid release was found, indicating a weak stimulation of PLA2 activity.

Discussion

A striking and unexpected result of the present work is the finding that high level of α2-adrenoceptor in rat foetal liver is due to expression of the α2B-subtype in haematopoietic cells of erythroblastic origin. As already suggested by *in situ* hybridization experiments (Wang & Limbird, 1997), our results indicate that a similar situation holds true in mouse but not in man. Together with previous data showing a huge discordance of expression levels in the kidney (Berkowitz *et al*., 1994), this observation confirms that major discrepancies exist in the pattern of α2B-adrenoceptor between rodents and other species. It is presumed that the pattern of the α2B-adrenergic receptor in rat is defined by regulatory sequences located in the 5′ and/or 3′ flanking region of the RNG gene. In contrast to that for the genes encoding other subtypes in rat (Handy *et al*., 1995; Saulnier-Blache *et al*., 1996) and man (Handy & Gavras, 1992; Schaak *et al*., 1997), no information is available yet about the promoter region of the α2B-subtype. However, we have recently cloned the 5′ non-coding region of the genes encoding human and rat α2B-adrenoceptor (GenBank accession number: AF005900 and AF366899, respectively). Whatever the subtype considered, the comparison of the sequences indicates a good conservation in the coding regions, (83–88% identity). Interestingly, the situation is quite different when the 5′ non coding regions are considered. Indeed, analysis of the sequence of the 1200 base-fragment upstream from ATG reveals a rather good conservation between rat and man for the α2A- (76%) or α2C-adrenoceptor (73%), but large divergence for the α2B-subtype (56% identity only). On the other hand, the promoter region of the mouse α2B-adrenoceptor (Chen *et al*., 1996) largely resembles the rat one (84% identity). This difference may be responsible for the divergence in the tissue

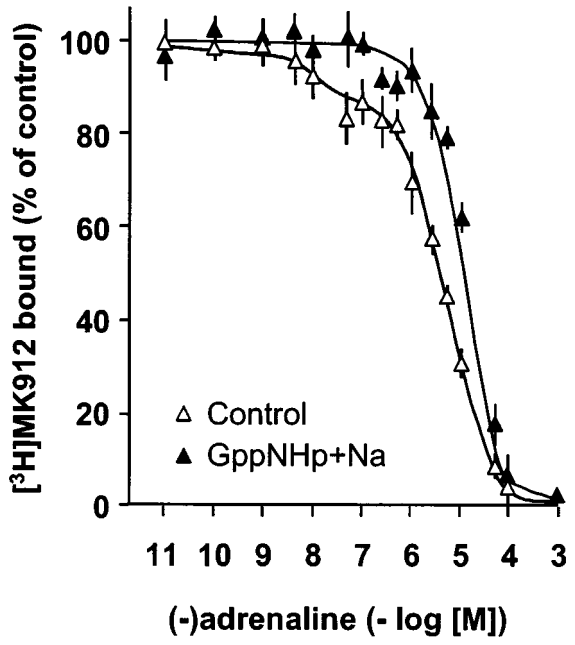

Figure 5 Receptor coupling to G protein. Membranes prepared from foetal liver were incubated in the presence of 3 nM [^{3}H]-MK912 and increasing concentrations of (−)adrenaline. The experiments were carried out either in the absence or in the presence of 10 μM GppNHp plus 100 mM NaCl. The amounts of bound radioligand are expressed as per cent of control and the presented data are means±s.e.mean from three independent experiments. Competition curve obtained in the absence but not in the presence of GppNHp plus Na^+ was better fitted according to a two-component inhibition model at $P<0.01$.

distribution of the α2B subtype between man and rodents. According to our results, the expression of the receptor in erythroblasts/reticulocytes gradually declines within the first weeks of life, so that the receptor becomes undetectable in blood from 21 day-old rat. Such an evolution resembles that previously observed in neonatal rat lung (Latifpour & Bylund, 1983). Although the present study does not provide the definitive demonstration, it is highly suggestive that expression of α2B-adrenoceptor in this tissue is solely due to the presence of blood. Consistent with this view, it is of note that substantial amounts of α2B-adrenoceptor were also found in spleen, at the early stage of life. Indeed, as depicted in Figure 7, [^{3}H]-RX821002 labelled with high affinity ($K_D=0.63\pm0.13$ nM) a single class of sites in adult spleen. In contrast, it clearly revealed two populations of receptors in spleen from newborn (day 3 of age; $K_{D,1}=0.45\pm0.16$ nM, $K_{D,2}=6.1\pm2.1$ nM). Inhibition experiments with oxymetazoline and prazosin demonstrated that α2-adrenergic receptivity is only of the α2A-subtype in adult, whereas it exists as a mixture of α2A and α2B in neonatal spleen. Moreover, conversion from the situation in newborn to that in adult is gradual. Similarly to what is observed in blood, expression of α2B-adrenoceptor in rat spleen declines with time, so that only trace amounts of α2B-subtype are detected at 3 weeks of age. As a consequence of the results from the present study, neonatal rat lung should not in future be considered as the tissue of reference for the expression of α2B-adrenoceptor subtype. Instead, foetal reticulocytes are proposed.

Also in agreement with previous findings showing that no appreciable specific binding of [^{3}H]-agonists was detectable in neonatal rat lung (Latifpour & Bylund, 1983), examination of receptor coupling in foetal liver (not shown) or in foetal

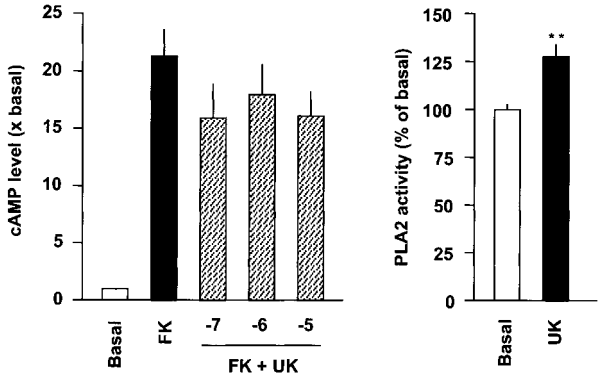

Figure 6 Effect of receptor stimulation on cyclic AMP level and arachidonic acid release. Foetal reticulocytes were separated by centrifuging blood collected from newborn rat (1 day of age) on Percoll gradient and extensively washed in HEPES-buffered DMEM (pH 7.4). Left panel: Reticulocytes were exposed to vehicle (basal), 10 μM forskolin (FK) and 10 μM forskolin plus 0.1, 1 or 10 μM UK14304 (FK+UK). After 20 min of incubation at 37°C, the reaction was stopped, cyclic AMP extracted and measured by RIA. Results are expressed as fold increase of basal cyclic AMP level and are means±s.e.mean from six experiments. Right panel: Reticulocytes were loaded with [^{3}H]-arachidonic acid (1 μCi ml^{-1}) for 1 h. They were then extensively washed in HEPES-buffered DMEM containing 0.2% fatty acid-free BSA and treated with 1 μM UK14304 (UK) or not (basal). Arachidonic acid release was measured as described in the Methods section. Results are expressed as percentage of control and are means±s.e.mean from three independent experiments with triplicate determinations.

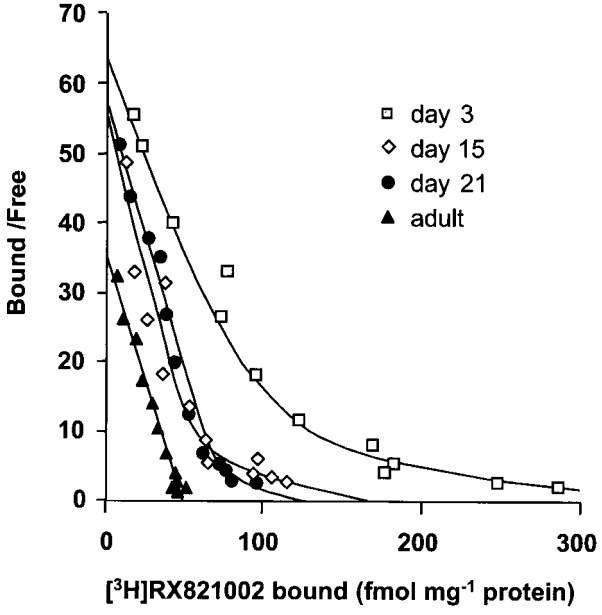

Figure 7 Expression of α2-adrenoceptors in the spleen of rat at different ages. Spleen was collected from newborn (3 days of age), young (15 or 21 days of age) and adult rats. Crude membranes were prepared and density of α2-adrenoceptors was examined using [^{3}H]-RX821002 as radioligand. The Scatchard plots are presented and results from computer analysis of the saturation isotherms are given in the text.

reticulocytes showed that a rather small fraction (~14%) of the receptor population is coupled to G-protein. However, as confirmed by direct study of [^{3}H]-clonidine binding, this fraction represents approximately 200 fmoles of potentially functional receptor per mg of protein. Such an amount is generally considered to be more than sufficient to elicit a biological response. Stimulation of receptor by UK14304 failed to inhibit significantly cyclic AMP production, but evoked an increase in arachidonic acid release. The failure to lower cyclic AMP production may appear surprising, but this result is consistent with data on transfected cells showing that α2B-adrenoceptor was less efficient than other subtypes in inhibiting adenylyl cyclase (Jansson *et al*., 1994). In certain cell-types, α2B-adrenoceptor has even been reported to raise cyclic AMP production through interaction with Gs (Pepperl & Regan, 1993). Such a paradoxical effect was however not observed in rat reticulocytes (not shown). On the other hand, the stimulation of arachidonic acid release agrees well with recent data in transfected CHO cells showing that, of the three subtypes, α2B-adrenoceptor was the most efficient in stimulating cPLA2 (Audubert *et al*., 1999). Further studies will be necessary to elucidate the consequences of receptor stimulation, but the present data already suggest that the receptor is functional.

The experimental induction of reticulocytosis in the adult rat did not cause the reappearance of the receptor, indicating that expression is restricted to a window of time and that its role, if any, occurs during the period of foetal haematopoiesis. It is obvious that a possible implication of the receptor in the proliferation and/or the differentiation of erythroid progenitor cells remains to be demonstrated. However, several lines of evidence support this hypothesis. First, disruption of the gene encoding tyrosine hydroxylase (Zhou *et al*., 1995) or dopamine β-hydroxylase (Thomas *et al*., 1995) results in embryo lethality, demonstrating that catecholamines play a crucial role during early mouse development. Of note, the liver of the deficient embryos is congested with blood. A similar anomaly is found in mice deficient in the transcription factor GATA3, which also have reduced noradrenaline levels (Lim *et al*., 2000). Second, α2-adrenoceptors are coupled to Gi-proteins which are important components in the mediation of intracellular signals triggered by haematopoietic growth factors. In erythroid precursor cells for instance, Gi2 is required for the regulation of voltage-independent Ca^{2+}-channels by erythropoietin (Miller *et al*., 1996). Furthermore, pertussis toxin impedes the erythropoietin-stimulated formation of colonies from erythroid progenitor cells isolated from rat foetal liver, suggesting that Gi proteins are involved in cell growth (Billat *et al*., 1991). Finally, it has been reported that α2B-knockout mice do not breed well and that, in clear contrast with mice lacking functional α2A- or α2C-subtype, heterozygote intercrosses produced fewer homozygotes than expected (Link *et al*., 1996). To our knowledge, runts have not yet been examined and the reason for lethality remains unknown. Given the present observations, it might be of great interest to examine whether α2B-knockout mice suffer dyserythropoiesis.

In conclusion, while α2A-adrenoceptor has recently been shown to play a role in the apoptotic process of mesenchyme taking place during digit formation (Wang & Limbird, 1997), the current observation strongly suggests a hitherto unappreciated role of α2B-adrenoceptor during embryonic erythropoiesis in rodents.

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