Rapid Desensitization of Neonatal Rat Liver β-Adrenergic Receptors

A Role for β-Adrenergic Receptor Kinase

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Abstract

Exposure of β-adrenergic receptors (BAR) to agonists often leads to a rapid loss of receptor responsiveness. The proposed mechanisms of such rapid receptor desensitization include receptor phosphorylation by either cAMP-dependent protein kinase or the specific β-adrenergic receptor kinase (BARK), leading to functional uncoupling from adenylyl cyclase and sequestration of the receptors away from the cell surface. To evaluate the physiological role of such mechanisms, we have investigated whether rapid regulation of BAR occurs in the neonatal rat liver immediately after birth, a physiological situation characterized by a dramatic but transient increase in plasma catecholamines. We have detected a rapid, transient uncoupling of liver plasma membrane BARs from adenylyl cyclase (corresponding to a desensitization of ~ 45%) within the first minutes of extrauterine life, followed by a transient sequestration of ~ 40% of the BARs away from the plasma membrane. In agreement with such pattern of desensitization, we have detected (by enzymatic and immunological assays) rapid changes in BARK specific activity in different neonatal rat liver subcellular fractions that take place within the same time frame of BAR uncoupling and sequestration. Our results provide new evidence on the potential role of BAR desensitization mechanisms in vivo and suggest that they are involved in modulating catecholamines actions at the moment of birth. Furthermore, our data indicate that in addition to its suggested role as a rapid modulator of adrenergic receptor function at synapse, rapid BARK-mediated receptor regulation may have functional relevance in other tissues in response to high circulating or local levels of agonists. (*J. Clin. Invest.* 1994. 93:937–943.) Key words: catecholamines • adenylyl cyclase • translocation • refractoriness • receptor regulation

Introduction

It is well known that cellular responsiveness to messenger stimulation is attenuated in the face of acute or sustained activation. This general biological phenomenon is based on the regulation of signal transduction systems and has been termed desensitization, tolerance, or refractoriness. A prototypic model for the study of the molecular mechanisms of desensitization has been the β-adrenergic receptor (BAR)[1] adenylyl cyclase system, which mediates a variety of important physiological functions of the catecholamines adrenaline and noradrenaline in different tissues (1–3). Work from several laboratories has shown that the molecular mechanisms underlying rapid or short-term desensitization of BAR involve both transient sequestration of receptors away from the plasma membrane and functional uncoupling of the receptor from stimulatory G protein. After long-term exposure to β agonists, other mechanisms involving changes in receptor synthesis and degradation also operate, usually leading to receptor downregulation (1–5).

The mechanisms involved in rapid receptor sequestration are poorly understood (6). On the other hand, the rapid functional uncoupling can be triggered by BAR phosphorylation by cAMP-dependent protein kinase (PKA) or β-adrenergic receptor kinase (BARK) (1, 2, 7). BARK is a cytoplasmic enzyme that specifically phosphorylates the agonist-occupied form of the BAR and other related G protein–coupled receptors (2, 7). BARK has been shown to rapidly translocate to the plasma membrane in response to the presence of β agonists in the extracellular medium (8–10); a role for βγ subunits of G proteins in BARK targeting to the membrane-bound receptor has recently been reported (11). Whereas PKA-mediated regulation directly leads to BAR uncoupling by phosphorylating an intracellular domain implicated in interaction with stimulatory G protein (3), phosphorylation by BARK promotes the binding to the receptor of another cytosolic protein, β-arrestin, which inhibits its coupling to α_s and adenylyl cyclase (12, 13). Recent data indicate that additional proteins, such as phosducin, may also participate in this complex regulatory network (14).

Several studies (15–19), have suggested that BARK-mediated regulatory mechanisms would be specially important in the presence of high levels of catecholamines, such as at neural synapses. On the contrary, PKA-mediated modulation, which has been shown to be a slower process (18), would predominate at lower agonist concentrations; i.e., in response to normal levels of circulating catecholamines. This interpretation is consistent with the fact that expression of BARK-1 mRNA is higher in the brain and highly innervated tissues (20), although this enzyme has also been reported to be expressed and functional in blood cells such as leukocytes (21).

To provide a better understanding of the physiological role of receptor regulatory processes, our laboratory is interested in investigating physiological situations in which such desensitization mechanisms might operate and could be detected. In this regard, it is noteworthy that the perinatal period is characterized by a dramatic surge in plasma catecholamines (22). In

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Received for publication 30 March 1993 and in revised form 4 October 1993.

J. Clin. Invest.

0021-9738/94/03/937/07 $2.00
Volume 93, March 1994, 937–943

1. *Abbreviations used in this paper:* BAR, β-adrenergic receptor; BARK, BAR kinase; DHA, dihydroalprenolol; GppNHp, guanyl-5'-yl-imidodiphosphate; P_3, microsomal internal membranes; PLSD, post hoc least significant difference; PM, plasma membrane; S_3, cytosol.

many species, including the rat, and to a somewhat lesser extent, humans, sympathetic innervation of autonomic organs is absent or nonfunctional at birth. Accordingly, in the fetus and neonate these messengers are released from the adrenal medulla and paraganglia in response to the physiological hypoxia associated with birth (23, 24), and promote critical metabolic, cardiovascular, thermogenic, and respiratory changes that are necessary to survive the transition to extrauterine life (22, 25). Such transient rise of catecholamines exceeds by an order of magnitude the messenger levels detected even in the most extreme or pathological circumstances in the adult; in asphyxiated neonates, even higher plasma catecholamine levels can be attained (22, 23). An intriguing question is whether such important changes in hormone levels could trigger processes of adrenergic receptor desensitization during the perinatal period. Thus, we hypothesized that rapid, catecholamine-induced changes in adenylyl cyclase coupling, β-adrenergic receptor compartmentation, and β-adrenergic receptor kinase subcellular distribution might be apparent in such physiological situation. In this report, we show that a rapid process of β-adrenergic receptor regulation takes place in the neonatal rat liver immediately after birth and explore its mechanisms.

Methods

Preparation of neonatal rat liver subcellular fractions. Term fetuses were delivered with intact placentas by rapid hysterectomy of cervically dislocated mothers (timed pregnant Wistar rats) at 22 d of gestation, exactly as described (24). Newborns were quickly detached after tying the umbilical cords and maintained at 37°C in a humidicrib with a water-saturated atmosphere and without feeding. Fetuses or newborn rats were killed by decapitation immediately after birth or $\leq$ 20 min after the induced delivery; it is worth noting that the time 0 actually corresponded to $\sim$ 45 s after the beginning of the hysterectomy procedure. Using exactly the same experimental protocol, neonatal plasma catecholamine levels of $\sim$ 50 nM ($\sim$ 8 ng/ml), have been reported at the moment of delivery, followed by a rapid decrease during the first 30 min of life (to $\sim$ 2 ng/ml), reaching levels of $\sim$ 1 ng/ml at 1 h after birth. All subsequent steps were performed at 4°C. Livers (pooled from six fetuses from three different mothers) were extracted, weighed, diced, and diluted in 4 vol of 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml benzamidine (buffer A) plus 250 mM sucrose, and homogenized with eight strokes of a motorized Teflon pestle. Subcellular fractionation was performed by established procedures (26, 27). Briefly, the supernatant of a low speed centrifugation (250 *g*, 4 min) was centrifuged at 3,000 *g* for 10 min to obtain a plasma membrane pellet. The centrifugation of this supernatant (10,000 *g*, 20 min) provided the crude mitochondrial pellet. Finally, the internal membranes or microsomal fraction (P_3) and the cytosol were obtained after a 250,000 *g* centrifugation (60 min) (TL-100; Beckman Instruments, Fullerton, CA). Subcellular fractions were characterized by electron microscopy as reported (reference 28, data not shown) and the distribution of marker enzymes for the plasma membrane (5′ nucleotidase), lysosomes (β-*N*-acetyl-glucoseaminidase) endoplasmic reticulum (glucose oxidase) and Golgi apparatus (α-mannosidase II) was measured. The plasma membrane fraction displayed a 6.3-fold increase in the specific activity of 5′ nucleotidase with respect to the microsomal fraction (P_3); P_3 fractions were enriched in endoplasmic reticulum, Golgi, and lysosomal markers, with little contamination of plasma membrane. All enzymatic markers were assayed as described (29). In other experiments, a different group of animals was used. 1 d-old rats were subjected to anoxia for 2–10 min exactly as previously described (30). After allowing 30 s of recovery, the asphyxiated newborn rats and the appropriate controls were killed by decapitation and liver subcellular fractions prepared exactly as described above.

Adenylyl cyclase assay. Determination of cyclase activity was performed essentially as described (31). Plasma membranes were washed three times in buffer A and the final pellet resuspended in 75 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 2 mM DTT to a protein concentration of 2–3 mg/ml. Approximately 80 μg of membrane protein were added to an incubation mixture containing 30 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.6 mM EDTA, 0.8 mM DTT, 10 mM creatinine-phosphate, 0.5 mg/ml creatinine-kinase, 100 μM ATP, 50 μM GTP, and various concentrations of isoproterenol, 10 mM NaF or 100 μM forskolin, in a final volume of 100 μl. After 15 min of incubation at 37°C, the reaction was stopped and cAMP quantified by radioimmunoassay (Amersham Corp., Arlington Heights, IL) as reported (32). Experimental data were analyzed as previously described (17). Basal activity was not affected by the different experimental conditions and was substracted from the values obtained in the presence of isoproterenol or activators. The resulting isoproterenol-induced stimulation was expressed as percent of the activity in the presence of 100 μM forskolin to normalize the data for effects that occur at the level of cyclase (i.e., heterologous desensitization). The extent of desensitization was calculated by measuring the loss of maximal stimulation by isoproterenol as described (17).

Binding studies. Plasma membranes (PM) or P_3 obtained as described above were washed three times and resuspended in buffer A at a protein concentration of 3 mg/ml. Total β-adrenergic receptor number was determined by incubating 300 μg of membranes for 30 min at 30°C with a saturating concentration of 2 nM [^{3}H]dihydroalprenolol ([^{3}H]DHA) (Amersham Corp.), using 50 μM (−)propanolol to define nonspecific binding; similar results were obtained when 100 μM (−) isoproterenol was used instead. For competition experiments, plasma membranes were incubated in the presence of the radioligand and different concentrations of (−) isoproterenol in a medium containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ascorbic acid, in the presence or absence of 200 μM guanyl-5′-yl-imidodiphosphate. Competition curves were fitted to both one- and two-site models using nonlinear least squares regression analysis and the quality of the fits compared by F test (33).

Determination of BARK. Since BARK has been shown to specifically phosphorylate rhodopsin in an agonist-dependent fashion (34), BARK activities in the different rat liver subcellular fractions were assessed by using purified urea-treated rod outer segments as substrate. This method has been previously used to determine BARK activity in cells and tissues and in BARK purification (9–11, 35). Rhodopsin kinase–free purified rod outer segments were prepared in the dark as reported (34), and aliquots containing 300–500 pmol of rhodopsin were incubated for 20 min at 30°C in the presence or absence of light in a medium containing 25 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 55 μM [γ-^{32}P]ATP (2–3 cpm/fmol) and extracts of rat liver subcellular fractions obtained from neonates at different times after delivery (final vol, 50 μl). Cytosol (S_3) containing soluble BARK activity was assayed directly. However, for determining BARK activity in membrane fractions, it first had to be extracted (10). PM or P_3 were washed once, resuspended in a small volume of buffer A, and treated for 15 min at 4°C with 200 mM NaCl in buffer A to extract peripheral proteins. Membranes were then pelleted and the supernatant containing the extracted proteins assayed for BARK activity as described above. Phosphorylation reactions were stopped by diluting 20-fold with ice-cold buffer A and centrifuging at 12,000 *g* for 15 min. The resultant pellets containing the phosphorylated rhodopsin were suspended in SDS-PAGE sample buffer and electrophoresed as described (10, 34), followed by autoradiography. BARK activity was quantitated by measuring the radioactivity associated to the excised rhodopsin band in the dried gel by Cerenkov spectroscopy, and normalized by the milligrams of protein present in the assay (9). In some experiments, we investigated the presence of BARK protein in cytosolic or membrane-extracted fractions by Western blotting. The different rat liver subcellular extracts ($\sim$ 200 μg of protein in SDS-PAGE sample buffer) were resolved by electrophoresis in 7.5% SDS-polyacrylamide gels and blotted to filters (Immobilon;

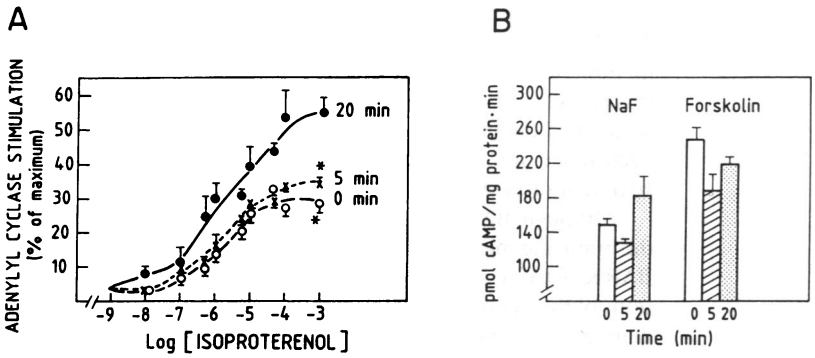

Figure 1. Desensitization of β-adrenergic receptors in the neonatal rat liver. (*A*) Adenylyl cyclase activities of plasma membranes obtained from the liver of neonatal rats killed immediately after birth (0 min, ○), 5 min after birth (×), or 20 min after delivery (●) was measured in the presence of various concentrations of (−) isoproterenol and expressed as percent of the activity in the presence of 100 μM forskolin, as detailed in Methods. The values for forskolin stimulation over basal at the different times are shown in (*B*). Adenylyl cyclase activities stimulated by 10 mM NaF or 100 μM forskolin in neonatal rat liver plasma membranes obtained from animals that were killed at the indicated times after delivery. In both *A* and *B*, results are means±SEM of three independent experiments performed in triplicate. There is a significant variation of maximal adenylyl cyclase stimulation by isoproterenol with time after delivery (one-way ANOVA, $F(2,9) = 9.55$, $P < 0.01$). Significant differences ($^*P < 0.05$) for maximal stimulation compared to that obtained at 20 min after delivery are shown (Fisher's PLSD).

Millipore Corp., Bedford, MA). Filters were probed with AB-9 (1:1,000), a polyclonal antibody raised against the recombinant BARK-1 protein expressed in Sf-9 cells (kindly provided by Dr. Jeffrey L. Benovic, Jefferson Institute, Philadelphia, PA), or AB-792 (1:40), an affinity-purified polyclonal antipeptide antibody raised in our laboratory against amino acids 533–544 of the bovine BARK-1 sequence (see reference 36). Filters were washed and developed using a chemiluminiscent method (Luminol; Amersham Corp.).

Statistical analysis. Data are expressed as mean±SEM. Statistical evaluation was performed using individual Student's *t* test, when appropriate, or one way ANOVA with Fisher's post hoc least significant difference test (PLSD), using the Statview program.

Results

As a first step to examine the existence of a possible BAR desensitization process in the perinatal rat liver, we investigated the stimulation of adenylyl cyclase activity by different concentrations of the β agonist isoproterenol in plasma membrane preparations obtained from animals killed at 0, 5, or 20 min after delivery. Fig. 1 *A* shows that the maximal response elicited by isoproterenol (normalized with respect to forskolin-induced stimulation) was markedly diminished at both the 0- and 5-min time points when compared to maximal stimulation at 20 min after delivery, corresponding to a desensitization of 40–45%. These results indicate that uncoupling of BAR from adenylyl cyclase occurs immediately after birth and that a recovery (resensitization) from such uncoupling proceeds thereafter. Such desensitization/recovery would correspond to β_2-adrenergic receptors, which is the predominant subtype in the neonatal rat liver. As shown in Fig. 1 *B*, only slight changes (12–17%) in adenylyl cyclase activation by NaF or forskolin (acting at the G protein and effector levels, respectively) are apparent in the same experimental conditions, and show a somewhat different, slower time course. For instance, the minimal stimulation by both activators is reached at 5 min after delivery, and forskolin-stimulated cyclase activity seems to be not fully recovered at 20 min after birth, as opposed to the isoproterenol-stimulated activity. These results suggest that the rapid regulation of this transduction system is preferentially taking place upstream of the G protein and the cyclase effector. Uncoupling at the receptor level is further suggested by analysis of radioligand binding agonist competition curves in the absence or in the presence of GppNHp, a nonhydrolisable analogue of GTP. In plasma membranes obtained from animals immediately after birth (Fig. 2 *A*), the percentage of receptors showing high affinity agonist binding [IC_{50} of (−) isoproterenol 1.3 nM] in the absence of GppNHP was only 31±4%; in the presence of GppNHp, a slight shift to the right of the competition curve occurs because of the conversion of most of the receptors to the low affinity state [IC_{50} of (−) isoproterenol 0.6 μM]. A markedly higher (about twofold) proportion of β-adrenergic receptors in the high affinity state (59±5%) was detected in membranes obtained at 20 min after delivery (Fig. 2 *B*), in agreement with the increased BAR-adenylyl cyclase coupling detected at this time point compared to the situation at birth (Fig. 2 *A*). The presence of GppNHp in the experiments performed with the 20 min plasma membranes also promoted the shift of receptors to a low affinity state (IC_{50} of (−) isoproterenol 0.8 μM).

Furthermore, a very rapid process of BAR sequestration can be monitored in rat liver during this early postnatal period. As assessed by [^{3}H]DHA binding, about a 25% decrease in the number of BAR in the plasma membrane can be detected within 2 min of delivery (Fig. 3, *closed circles*), and by 5–10 min of life a significant decrease of ~ 40% with respect to the

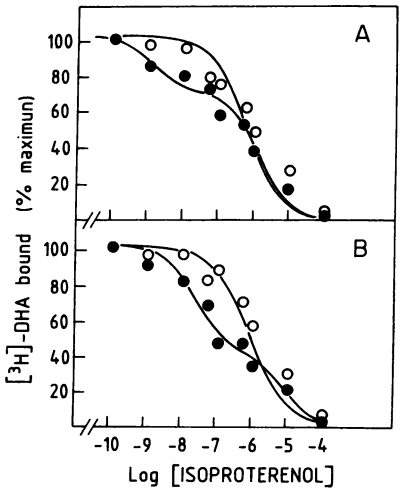

Figure 2. Functional coupling of β-adrenergic receptors in rat liver plasma membranes derived from animals killed at birth (*A*) or 20 min after delivery (*B*). Membranes were incubated with increasing concentrations of the β agonist (−) isoproterenol to compete for the binding to the BAR with the radiolabelled antagonist [^{3}H]DHA (see Methods) in the absence (●) or presence (○) of 200 μM GppNHp. The data shown are the means of three independent experiments with triplicate determinations. Data were fitted and analyzed as described in Methods. Isoproterenol competition curves in the absence of GppNHp were best described by a two-site model at both time points (F test, $P < 0.05$).

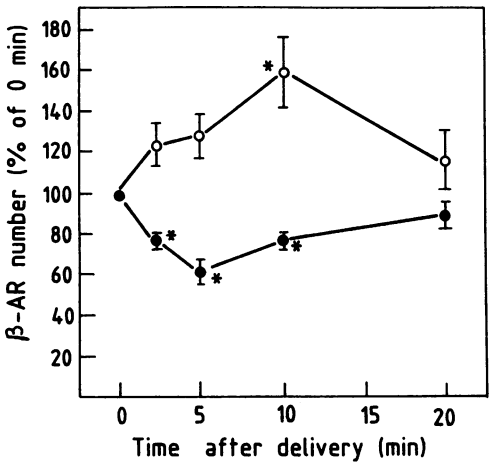

Figure 3. Time course of rat liver β-adrenergic receptor redistribution during the first minutes after delivery. Plasma membrane (●) and internal membranes (○) fractions were obtained as described in Methods from the liver of neonatal rats sacrificed at the indicated times after delivery. Total β-adrenergic receptor number in each fraction was estimated by radioligand binding as detailed in Methods. The values obtained immediately after birth (18±2 fmol/mg protein for the internal membranes and 36±3 fmol/mg protein for plasma membranes) were taken as 100%. Data are means±SEM of four to nine experiments assayed in triplicate. There is a significant variation of β-adrenergic receptor number in the plasma membrane with time after delivery (one-way ANOVA, $P(4, 27) = 5.74$, $P < 0.01$). Significant differences ($^*P < 0.05$) with respect to the 0-min time points are shown (Fisher's PLSD).

levels at birth is attained. Conversely, an increase in β-adrenergic receptors present in internal, microsomal membranes is noted within the same time frame (Fig. 3, *open circles*), thus indicating the existence of a sequestration process. Such redistribution of BAR is transient, since the internalized receptors gradually return to the plasma membrane, as indicated by the data obtained at 20 min after delivery in both plasma and internal membranes (Fig. 3).

The receptor uncoupling and sequestration data strongly suggested that a very rapid process of predominantly homologous (i.e., agonist-specific) BAR desensitization was taking place in the neonatal rat liver immediately after birth. Homologous desensitization of BAR has been reported to involve specific receptor phosphorylation by the BARK after agonist-induced kinase translocation from the cytoplasm to the plasma membrane (1, 2). To investigate a possible role for BARK in this process of BAR desensitization detected in neonatal liver, we studied the presence of BARK in perinatal rat liver and the occurrence of changes in BARK subcellular distribution (i.e., BARK translocation).

Although BARK expression has been mostly ascribed to the brain and highly innervated tissues (20) we have found that BARK activity is clearly present in different subcellular fractions of the neonatal liver, as demonstrated by the light-dependent phosphorylation of rhodopsin (Fig. 4 *A*). Antibodies raised against recombinant BARK-1 specifically detected in extracts from the different subcellular fractions a band of ~ 80 kD, which comigrates with recombinant bovine BARK-1 expressed in Sf-9 cells (Fig. 4 *B*). The same results are obtained with antipeptide antibodies generated against a bovine BARK-1 sequence (data not shown, see also reference 36). Interestingly, important changes in BARK specific activity in the different rat liver subcellular fractions are detected within the same time frame of BAR desensitization. As shown in Fig. 5, BARK-specific activity transiently increases in the plasma membrane, reaching a peak at 5 min of life and decreasing thereafter, whereas it slightly decreases in the cytosol and significantly increases in the internal membranes fraction during the first 20 min after delivery. It should be noted that BARK-specific activity in the latter fraction is slightly higher than that in plasma membrane and more than 10-fold higher than that of cytosol (see legend to Fig. 5). The subcellular distribution of total BARK activity is shown in Table I. Soluble BARK accounts for ~ 30% of total activity at birth, and tends to decrease with time, whereas a significant increase is noted in the kinase associated to microsomal membranes (from 50 to 65%); slight increases are noted in the size of the BARK pool already associated to plasma membrane fractions at the moment of birth. It is interesting to note that the increase in total BARK activity noted in the plasma membrane and microsomal fractions is higher than the parallel decrease in soluble BARK, thus suggesting the existence of additional factors that may modulate BARK activity (11).

To confirm that catecholamines could promote a very rapid translocation of BARK in rat liver, we investigated the effect of subjecting 1-d-old rats to brief periods of anoxia on the subcellular distribution of the enzyme. 1 d-old rats are still able

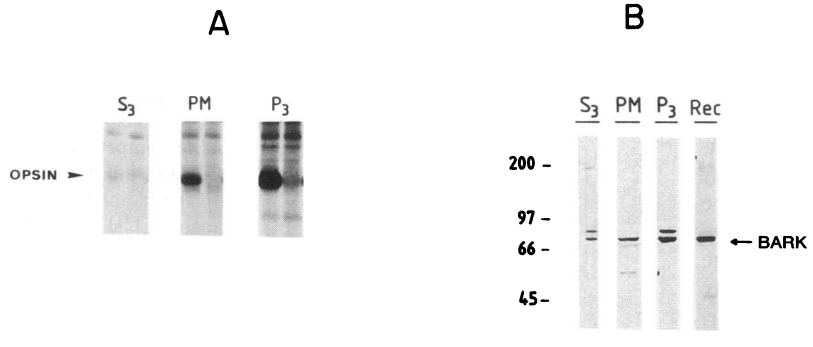

Figure 4. Presence of β-adrenergic receptor kinase in the neonatal rat liver. (*A*) S_3, PM, or P_3 were obtained from the liver of neonatal rats killed 10 min after delivery, and BARK activity was extracted from membrane fractions as detailed in Methods. The different extracts were incubated under phosphorylating conditions with urea-treated purified rod outer segments either in the presence (left lanes under S_3, PM, and P_3) or absence of light (right lanes), followed by SDS-PAGE and autoradiography. The migration of the opsin band (assessed by Coomassie blue staining) is marked with an arrow. The autoradiogram is representative of three experiments. (*B*) S_3, PM, or P_3 extracts from neonatal rat liver were prepared as described in *A* and Methods. These extracts and recombinant BARK-1 expressed in Sf-9 cells (*Rec*) were resolved by 7.5% SDS-PAGE, blotted and probed with a polyclonal antibody raised against recombinant bovine BARK-1 (this figure) or with an affinity-purified anti-peptide antibody raised against a bovine BARK-1 sequence (data not shown). The antibodies recognize a band (marked with an arrow) with an apparent relative molecular mass of ~ 80 kD, similar to that reported for BARK-1 (20), which comigrates with recombinant BARK (*Rec*).

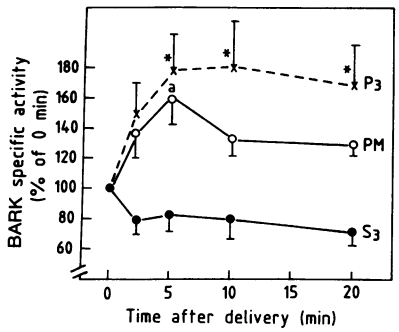

Figure 5. Changes in the BARK activity associated to different liver subcellular fractions during the first minutes after delivery. Extracts were obtained as in Fig. 4 from the liver of rats sacrificed at the indicated times after delivery and BARK activity measured in an assay based in light-dependent phosphorylation of rhodopsin as described (9, 10). BARK specific activity was quantitated as detailed in Methods. The values obtained immediately after birth were taken as 100% (6±1, 59±8 and 64±6 pmol of phosphate incorporated into rhodopsin per milligram of protein for S_3 [●], PM [○], and P_3 [×] fractions, respectively). Results are means±SEM of four independent experiments performed in triplicate. There is a significant variation of microsomal BARK activity with time after delivery (one-way ANOVA, $F(4, 17) = 2.98$, $P < 0.05$). (*a*) significant difference ($P < 0.05$) compared to the 0-min time point (Student's *t* test); *Significant difference compared to the 0-min time point (Fisher's PLSD).

to release huge amounts of catecholamines from the adrenal medulla in response to hypoxia (23). Preliminary results using this experimental system indicate a marked increase (~ 2.5-fold) in liver plasma and microsomal membrane BARK specific activity after 2 min of anoxia as compared to control animals (data not shown). These results suggest that an increase in circulating catecholamines as a result of either induced delivery or experimental hypoxia can promote very rapid changes in the specific activity of BARK in different subcellular fractions of the neonatal rat liver, the time course of such changes being consistent with a role for BARK-mediated phosphorylation in the regulation of BAR immediately after birth.

Discussion

We have investigated whether a physiological process of rapid β-adrenergic receptor regulation takes place during the perinatal period, which is characterized by a dramatic, transient increase in plasma catecholamines triggered by the hypoxia associated with delivery (22, 25). Our results indicate that a rapid uncoupling of liver plasma membrane BAR from adenylyl cyclase occurs immediately after birth, followed by a recovery or resensitization detected at 20 min after delivery. The overall pattern and the estimated extent of desensitization (40–45%) are similar to those described in cellular models of agonist-specific BAR desensitization (2, 10, 17, 18, 37). Only slight changes in the adenylyl cyclase response to activators at the level of G proteins or the effector itself are detected. This small heterologous component is probably caused by PKA-mediated regulation of these proteins, as also suggested by the slower time course of such effects when compared to BAR uncoupling (18). In agreement with a process of predominantly homologous BAR desensitization, binding studies indicated that plasma membrane adrenergic receptors were poorly coupled to G proteins immediately after birth, as has been described in the newborn rabbit liver (38) and in the lungs of adult rats infused with isoproterenol (31).

Furthermore, a very rapid, transient sequestration of plasma membrane receptors was noted in our experimental system shortly after birth. Receptors seem to be translocated to internal membrane compartments, as has been reported with a similar time course in cultured cells exposed to β agonists (6, 10, 39–41) or in the lung of isoproterenol-treated adult rats (31). The exact intracellular fate of BAR and the sequestration-recycling mechanisms are poorly understood, although recent data suggest the implication of endosomes (41). It is worth noting that in our model, as in other experimental situations, the adenylyl cyclase response is clearly diminished before the maximal internalization is reached, thus reinforcing the hypothesis that BAR uncoupling precedes sequestration (2, 3, 6, 39). Moreover, it is important to stress the good temporal correlation between the recycling of internalized BAR back to the plasma membrane and the recovery of isoproterenol-stimulated adenylyl cyclase activity observed 20 min after birth. It has been recently suggested that the main role of sequestration would be to allow the dephosphorylation and recycling of uncoupled, phosphorylated plasma membrane receptors (2, 6, 42). Our results are consistent with such suggestion, and rein-

Table I. Subcellular Distribution of Total BARK Activity in the Newborn Rat Liver

	Time after delivery					
	0 min		5 min		20 min	
	Total fraction activity	Percent of total tissue activity	Total fraction activity	Percent of total tissue activity	Total fraction activity	Percent of total tissue activity
	pmol/g of tissue		*pmol/g of tissue*		*pmol/g of tissue*	
Cytosol (S3)	114±21	31±4	100±4	23±3	91±1.6	20±4
Plasma membrane fractions	42±2	14±2	63.5±2	17±1	52±12	62±2
Microsomal fraction (P_3)	161±9	50±7	241±13*	64±5	210±37	59±5

Total BARK activity in each fraction and time point was calculated in each independent experiment from the specific activity values (expressed in picomoles of phosphate incorporated into rhodopsin per milligram of protein), taking into account the volume of each fraction obtained per gram of tissue (4 ml of cytosol, 1.5 ml of resuspended plasma membrane fractions, and 1 ml of resuspended microsomal fraction) and the protein concentration of the samples used in the rhodopsin phosphorylation assay (5–6 mg/ml cytosol, 0.6–0.8 mg/ml extracted plasma membranes, and 2–2.5 mg/ml extracted microsomal membranes). Results are means±SEM of three to four independent experiments performed in triplicate. The percent of total tissue activity at different times after delivery was determined in each experiment taking the sum of total BARK activities as 100%. The results are means±SEM of three to four independent experiments. There is a significant variation of microsomal BARK activity with time after delivery (one-way ANOVA, $P < 0.05$). * Significant difference ($P < 0.05$) compared to the 0-min time point (Fisher's PLSD).

force the fact that we are observing a desensitization/resensitization process vs a possible postnatal supersensitization of β-adrenergic receptors.

BARK-mediated receptor phosphorylation has been suggested to play a key role in the mechanisms of agonist-specific, homologous BAR desensitization. Thus, we next sought to establish whether this kinase was present in the neonatal rat liver and if BARK translocation and activation was taking place within the same time frame of the observed process of BAR desensitization. The presence of BARK activity in the neonatal rat liver is demonstrated by the fact that extracts from different subcellular fractions are able to phosphorylate rhodopsin in a light-dependent way. Such activity is inhibited by low concentrations of Zn^{2+} or heparin (data not shown), which are known negative modulators of BARK (18, 35). Furthermore, two different antibodies generated against bovine BARK-1 (20, 36) recognized a band of 80 kD in neonatal liver extracts, thus indicating that this kinase isoform or a closely related, cross-reacting isoenzyme is expressed in this tissue.

With regard to BARK translocation, our results indicate the occurence of significant changes in BARK specific activity in plasma membrane and other liver subcellular fractions with a close temporal relationship to the process of BAR uncoupling and sequestration. The extent of such changes in specific activity are similar to those reported for BARK translocation in response to β agonists in C6 glioma cells (10) or human peripheral blood leukocytes (21). Since β-adrenergic receptors are already uncoupled from adenylyl cyclase at the moment of birth (time point 0) and BARK translocation is required for agonist-induced BAR phosphorylation and homologous receptor desensitization, it could be argued that significant BARK translocation (i.e., an increase in plasma membrane BARK specific activity) should already had occurred at this point. The question is difficult to address in our in vivo experimental model. First, no control animals (i.e., animals unaffected by the surge of plasma catecholamines at delivery) are available to establish the basal values of BARK-specific activity in the different cellular fractions. Second, since as explained in Methods, time point 0 corresponds to 45 s after initiating the surgical delivery of the fetuses, and BARK translocation and receptor phosphorylation in response to β agonists occurs very rapidly in A431 cells (18) and in C6 glioma cells (10), it is likely that 45 s after delivery, BARK translocation has already taken place to a significant extent, and that we detect only a fraction of the BARK "peak." The rapidity and relatively higher extent of BARK translocation with respect to adequate controls observed in 1-d-old rats subjected to anoxia are also consistent with this interpretation. In fact, Table I indicates that a significant amount of total BARK activity is already present in plasma membrane fractions immediately after birth. It still remains to be established whether such changes in BARK subcellular localization are caused by actual protein translocation, kinase activation, or a combination of both mechanisms.

In conclusion, our results are consistent with rapid BARK translocation preceding BAR uncoupling and sequestration, thus strongly suggesting a key role for the kinase in this physiological process of BAR regulation triggered by the surge in plasma catecholamines associated with birth. However, given the fact that BARK can regulate other G protein–coupled receptors (2, 7, 9, 21), the possibility that part of BARK translocation is caused by the concurrent activation of other receptors different from BAR cannot be completely ruled out.

Previous in vivo studies have detected BAR desensitization and downregulation in different tissues after long-term infusion with β agonists or chronic pathological increases in circulating catecholamines, such as in heart failure, hypertension, or pheochromocytoma (43–45). On the contrary, our experimental model is based in transient, physiological changes in catecholamine concentrations. The rapid time course of desensitization that we observe correlates well with clinical observations in other systems showing reduced sensitivity to β agonists within minutes of parenteral administration of antiasthmatic drugs (reference 30 and references therein). Our results are also consistent with the refractoriness to β-adrenergic stimulation of glycogenolysis in the neonatal rabbit and rat liver in the first hours of life, although additional factors may be involved in order to explain such long-term unresponsiveness (24, 37, 46, 47). Further studies will be needed in order to ascertain the physiological role of such very rapid desensitization of BAR and to identify possible functional correlates (gene expression, induction of mitochondrial maturation) for such transient liver BAR activation at birth, in the context of the catecholamines actions during the perinatal period. A different sensitivity to desensitization would allow the tissues and cells to respond to a different extent or with variable rates to the same surge in plasma catecholamines. Thus, it is tempting to suggest that these mechanisms of receptor regulation play a key role in limiting and modulating the extent, kind and time frame of catecholamine actions during the perinatal period.

In conclusion, our results suggest a functional relevance for BARK-mediated phosphorylation and uncoupling of BAR in vivo. In agreement with recent results (21), our data indicate a physiological role for this kinase in peripheral tissues in response to plasma catecholamines, in addition to its suggested role as rapid regulator of adrenergic receptor function in the brain and other highly innvervated tissues, where cells are exposed to high and rapidly changing concentrations of catecholamines released at the synapse (2, 18).

Acknowledgments

The authors thank Dr. R. J. Lefkowitz and Dr. I. Sandoval for critical reading of the manuscript, Dr. J. L. Benovic for providing recombinant BARK-1, the Ab-9 polyclonal antibody and helpful suggestions, and Dr. J. M. Cuezva for his comments. C. Murga and P. Penela are gratefully acknowledged for help in raising and characterizing a BARK-1 antipeptide antibody, Prof. F. Mayor for continous encouragement, C. San Martín for the electron microscopy studies, Dr. Vázquez for help with the statistical analysis, and Mrs. M. Sanz for skillful secretarial assistance.

This work was supported by Comision Interministerial Ciencia y Tecnología grants PM890060 and PB92-0135, CAM C105/91, Boehringer Ingelheim, and Fundación Ramón Areces. I. Garcia-Higuera was recipient of predoctoral fellowships from Andrómaco and the Basque Government.

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