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Effects of the alkylating agent EEDQ on regulatory G proteins and recovery of agonist and antagonist α_2-adrenoceptor binding sites in rat brain

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Abstract

The aim of this study was to assess the effect of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)-induced α_2-adrenoceptor inactivation on regulatory G proteins and the recovery of agonist and antagonist binding sites. EEDQ induced a rapid increase in the abundance of rat brain cortical $G\alpha_{i1/2}$ proteins (30% at 6 h) which reached a maximum at 4 days (45%) and which then slowly returned (7–30 days) to control values. EEDQ did not alter significantly the levels of $G\alpha_{i3}$ and $G\alpha_o$ proteins. By using the standard monoexponential model, the analysis of the recovery of α_2-adrenoceptor density (6 h–30 days) with [^{3}H]UK 14304 (bromoxidine) and [^{3}H]RX 821002 (2-metoxy idazoxan) in the cerebral cortex did not reveal differences in receptor turnover parameters. However, the recovery of [^{3}H]UK 14304 binding fitted best to a new biphasic recovery model, suggesting the existence of two distinct phases of recovery of agonist sites (r_1 and $r_2 = 15.7$ and 7.4 fmol mg protein^{-1} day^{-1}; k_1 and $k_2 = 0.51$ and 0.25 day^{-1}; $(t_{1/2})_1$ and $(t_{1/2})_2 = 1.4$ and 2.7 days). In contrast, the recovery of [^{3}H]RX 821002 antagonist sites did not fit to the biphasic model ($r = 8.1$, $k = 0.14$, $t_{1/2} = 4.9$). Because agonist binding requires coupling to G proteins, the present results suggest that the rapid over-expression of $G\alpha_{i1/2}$ proteins induced by EEDQ is related to the biphasic recovery of [^{3}H]UK 14304 binding. The possible implication of the faster recovery of α_2-adrenoceptor function after EEDQ inactivation is discussed.

Keywords: α_2-Adrenoceptor turnover; [^{3}H]UK 14304 (bromoxidine); [^{3}H]RX 821002 (2-methoxy idazoxan); G protein; EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline)

1. Introduction

The irreversible inactivation of receptors and subsequent evaluation of their reappearance has been the approach most widely used to assess the turnover of adrenoceptors in vivo and in vitro (Mahan et al., 1987). *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) is a peptide-coupling agent that inactivates in vivo and in vitro several types of neurotransmitter receptors (Meller et al., 1988). Its ability to inactivate irreversibly various types of receptors probably involves activation of carboxyl groups, at or near the active binding site, for attack by a sterically accessible nucleophilic moiety (Belleau et al., 1969). Thus, EEDQ provides a useful tool for the investigation of receptor turnover as well as the relationship between receptor density and behavioural or physiological function. In vivo low doses (< 2 mg kg^{-1}) of EEDQ appear to be rather selective for α-adrenoceptors, especially for the α_2-adrenoceptor class (Meller et al., 1988; Pilc et al., 1992). Moreover, EEDQ does not discriminate between α_2-adrenoceptor subtypes (Barturen and García-Sevilla, 1992).

The α_2-adrenoceptors are a heterogenous group of G protein-coupled receptors that mediate many of the biological effects of catecholamines (Bylund et al., 1994; Lanier and Limbird, 1997). In the central nervous system, α_2-adrenoceptors play an important physiological role in the regulation of transmitter release from noradrenergic nerve terminals (Starke, 1987; Langer and Lehmann, 1988). In human and rat brains, the noradrenaline-regulating α_2-autoreceptors belong to the orthologue $\alpha_{2A/D}$-subtype (Raiteri

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et al., 1992; Starke et al., 1995), which is the predominant receptor in the cerebral cortex of both species. Other physiological functions served by the α_{2A}-, α_{2B}- and α_{2C}-subtypes have been elucidated (Esteban et al., 1996; Link et al., 1996; MacMillan et al., 1996; MacDonald et al., 1997).

α_2-Adrenoceptors have been shown to express two conformational states of high and low affinity for agonists (Hoffman et al., 1980; Asakura et al., 1985) but only the high-affinity conformation of the receptor mediates the inhibition of adenylyl cyclase (Nomura et al., 1987; Thomsen et al., 1988) and the induction of functional responses (García-Sevilla et al., 1988). In most cells types, agonist occupancy of α_2-adrenoceptors leads to inhibition of adenylyl cyclase through activation of specific G_i ($G\alpha_{i1/2/3}$) proteins. In the brain, the most abundant G proteins are G_o (Neer, 1990) and $G\alpha_{i1/2}$ (Goldsmith et al., 1988). For the α_2-adrenoceptors the factors that determine signal specificity through preferred G_i proteins are poorly understood, e.g., the α_{2A}-subtype can be coupled to several types of inhibitory G proteins ($G\alpha_{i1/2/3}$ and $G\alpha_o$) (Gerhardt and Neubig, 1991; Kurose et al., 1991; Eason et al., 1992; Okuma and Reisine, 1992). However, the prototypical α_{2A}-adrenoceptor of human platelets was shown to induce the inhibition of adenylyl cyclase through activation of $G\alpha_{i2}$, but not $G\alpha_{i3}$, proteins (Simonds et al., 1989), as was the case in other cells and tissues (Remaury et al., 1993; Valet et al., 1993). It, therefore, appears that the preferred functional $G\alpha_i$ protein for the α_{2A}-adrenoceptor is the $G\alpha_{i2}$ protein (McClue et al., 1992; Grassie and Milligan, 1995).

Against this background, the aim of the present study was to assess the effect of EEDQ-induced α_2-adrenoceptor inactivation on putatively coupled regulatory G_i proteins and agonist and antagonist α_2-adrenoceptor binding sites during the process of recovery of receptor density.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at 20 ± 2°C with a 12-h light/dark cycle. Rats were killed by decapitation, the brains were rapidly removed and the frontal and parieto-occipital cortices were dissected on ice and stored at −70°C until assayed.

2.2. EEDQ treatment

EEDQ (1.6 mg kg^{-1}) was dissolved in ethanol and then diluted sequentially with propyleneglycol and purified water (final ratio, 1:1:2, v/v/v). It was administered intraperitoneally (i.p.) in a single dose. Rats were killed 6 and 15 h, and 1, 2, 4, 7, 9, 14, 21, 26 and 30 days after EEDQ administration to evaluate the effect on G_i protein levels and recovery of brain α_2-adrenoceptor density, which allowed estimation of receptor turnover parameters. Control rats received i.p. a single injection of drug-vehicle to assess the steady-state levels of G_i proteins and density of α_2-adrenoceptors. These experiments in rats were performed according to the guidelines of the University of Balearic Islands.

2.3. Immunoblot analysis of G protein subunits

Preparation of cortical membranes (P2 membrane fraction), immunoblot analysis of specific G protein subunits and quantitation of specific immunoreactivity were done as described previously (Escribá et al., 1994; Ventayol et al., 1997). Briefly, solubilized G proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (Western blotting) and then labelled with specific antibodies. For G protein immunodetection, the nitrocellulose membranes were incubated overnight at 4°C in a blocking solution that contained the appropriate amount of the primary antibody: anti-$G\alpha_{i1/2}$ (AS/7) at a dilution of 1:7000, anti-$G\alpha_{i3}$ (EC/2) at a dilution of 1:3000 and anti-$G\alpha_o$ (GC/2) at a dilution of 1:4000. The secondary antibody, horseradish peroxidase-labelled donkey anti-rabbit immunoglobulin G, was incubated at a dilution of 1:5000 in blocking solution at room temperature for 2 h. Immunoreactivity was detected with the Enhanced Chemiluminescence Western Blot Detection system (Amersham), followed by exposure to Hyperfilm ECL film for 1 to 10 min. The film was scanned in the image analyzer Bio Image (Millipore, Ann Arbor, MI). The antisera used labelled bands with relative molecular masses of 40–41 kDa ($G\alpha_{i1/2}$ and $G\alpha_{i3}$) and 39–40 kDa ($G\alpha_o$), which was in good agreement with previous findings in human and rat brains (Escribá et al., 1994; Ventayol et al., 1997). The quantitation of specific immunoreactivity was done as described previously (Escribá et al., 1994), using appropriate standards curves (i.e., total protein loaded vs. integrated optical density, IOD), which consisted of at least four different protein contents (the protein was from naive rats), all loaded on the same gel, resulting in linear relationships in the range of protein content used (see Fig. 1). If a known amount of membrane protein from a test sample was loaded in the gel well (PR, real amount), the percent change with respect to control samples was calculated as the ratio between the amount of protein corresponding to the IOD value of the test sample interpolated from the control standard curve (PT, theoretical amount) and PR. Thus, the percent change = (PT/PR) × 100, where this value is 100 for a control sample used as the standard.

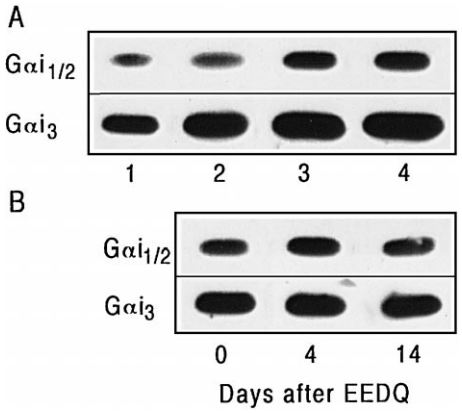

Fig. 1. Representative immunoblots of $G\alpha_{i1/2}$ and $G\alpha_{i3}$ protein subunits in the rat cerebral cortex. (A) Relation between G protein signal intensity and protein content. The amount of total protein (in μg) loaded per gel well was 4.5 (lane 1), 8.9 (lane 2), 13.5 (lane 3) and 18.6 (lane 4) for $G\alpha_{i1/2}$; and 2.6 (lane 1), 5.2 (lane 2), 7.8 (lane 3) and 10.4 (lane 4) for $G\alpha_{i3}$. The corresponding IOD values (arbitrary units) measured by image analysis were: 0.7, 1.5, 2.5, and 2.7 for $G\alpha_{i1/2}$ and 3.0, 5.4, 7.1 and 8.0 for $G\alpha_{i3}$. (B) Representative immunoblots of specific G protein subunits from rats injected with drug-vehicle or a single dose (1.6 mg kg^{-1}, i.p.) of EEDQ for 4 and 14 days. The amount of total protein loaded per gel well was 9.5 μg for $G\alpha_{i1/2}$ and 4.5 μg for $G\alpha_{i3}$. The signal intensity of the immunoreactive bands (IOD) was 1.8, 2.5 and 1.3 for $G\alpha_{i1/2}$, and 4.2, 4.3 and 4.2 for $G\alpha_{i3}$. Samples in (A) and (B) are from the same corresponding gels.

For the various G proteins the mean inter-assay coefficients of variation were 11–16%.

2.4. [^{3}H]UK 14304 and [^{3}H]RX 821002 binding to brain membranes

The specific binding of [^{3}H]UK 14304 to parieto-occipital cortical membranes was used as a biochemical index to quantify the density of α_2-adrenoceptor agonist binding sites. The agonist UK 14304 has different selectivity for the various α_2-adrenoceptor subtypes (K_d = 5.3, 22.0 and 2.0 nM for rat recombinant α_{2A}-, α_{2B}- and α_{2C}-subtypes, respectively (Tunstall et al., 1996); K_d = 3.7, 512 and 120 nM for α_{2A}-, α_{2B}- and α_{2C}-subtypes, respectively, in transfected S115 cells (MacDonald et al., 1997). Similarly, the selective antagonist [^{3}H]RX 821002 was used to quantify the density of α_2-adrenoceptor antagonist binding sites. The antagonist RX 821002 has similar affinity for the α_{2A}-, α_{2B}- and α_{2C}-subtypes (K_d = 1.4, 5.4 and 1.6 nM) (Tunstall et al., 1996).

Preparation of cortical membranes (P_2 membrane fraction) and [^{3}H]radioligand binding assays were done as described previously (Ribas et al., 1993). Briefly, total [^{3}H]UK 14304 binding was measured in 1.1-ml aliquots (50 mM Tris–HCl, 0.1 mM $MnCl_2$, 0.1% ascorbic acid, pH 7.7) of cortical membranes which were incubated for 60 min at 25°C with eight concentrations of [^{3}H]UK 14304 (6×10^{-11} to 8×10^{-9} M). In some experiments, the binding of [^{3}H]UK 14304 was measured without $MnCl_2$ in the incubation medium. Total [^{3}H]RX 821002 binding was also measured in 1.1-ml aliquots (50 mM Tris–HCl, 0.1% ascorbic acid, pH 7.5) of membranes which were incubated for 30 min at 25°C with eight concentrations of [^{3}H]RX 821002 (6×10^{-11} to 8×10^{-9} M). For both radioligands, non-specific binding (about 15% at K_d values) was determined in the presence of 10^{-5} M (−)-adrenaline.

Drug competition studies were performed with cortical membranes that were incubated with 10^{-9} M of [^{3}H]RX 821002 and 0.3×10^{-6} M of serotonin (5-hydroxytryptamine, 5-HT) to prevent the binding of the radioligand to the 5-HT_{1A} receptor (Vauquelin et al., 1990; Miralles et al., 1993a) in the absence or presence of various concentrations of the competing drugs (BRL 44408 and ARC 239, 3.3×10^{-11} to 10^{-3} M; 22 concentrations).

Membrane-bound [^{3}H]radioligands were quantified as described previously (Ribas et al., 1993). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Analyses of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding model were performed by computer-assisted non-linear analysis of untransformed data, using the EBDA-LIGAND programs (Munson and Rodbard, 1980).

2.5. Analyses of the recovery of α_2-adrenoceptor binding sites after EEDQ

The best way to analyze and compare the recovery of agonist and antagonist binding site densities is to use turnover functions. Data for the recovery (all individual experiments together) of brain α_2-adrenoceptor density after irreversible inactivation by EEDQ were analyzed (Barturen and García-Sevilla, 1992; Ribas et al., 1993) according to a monoexponential model based on two implicit assumptions (Mauger et al., 1982), namely that (1) the rate of receptor appearance is constant during the repopulation period (i.e., zero-order process) and (2) receptor disappearance is proportional to the density of receptors at any time (i.e., first-order process). Exponential recovery data were fitted, using the simple non-linear least-squares fitting program GraFit (Leatherbarrow, 1990), to the equation:

$$R_t = r/k(1 - e^{-kt}) \quad (1)$$

where R_t is expressed as fmol mg^{-1} protein and represents the receptor number at a given discrete time t; r is the rate constant of receptor appearance expressed as fmol mg $protein^{-1}$ day^{-1}, and k is the rate constant of receptor disappearance (in units of day^{-1}) which allows estimation of the apparent half-life of the receptor ($t_{1/2} = \ln 2/k$). In the present model the ratio r/k represents the density of

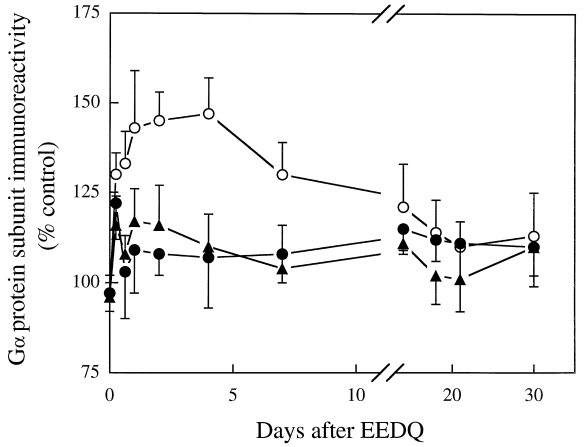

Fig. 2. Time-course for the immunoreactivity of $G\alpha_{i1/2}$ (○), $G\alpha_{i3}$ (●) and $G\alpha_{o}$ (▲) protein subunits after EEDQ-induced receptor inactivation in the rat frontal cortex. Rats were injected with the irreversible antagonist EEDQ (1.6 mg kg^{-1}, i.p.) and killed after various periods of time (6 and 15 h, and 1, 2, 4, 7, 14, 18, 21 and 30 days). Immunoblot analysis and quantitation of specific immunoreactivity were performed as described in Section 2 (see also Fig. 1). Data are means ± S.E.M. of three to five experiments per group performed in triplicate with an animal per experiment, and expressed as percentage of control. One-way ANOVA detected significant differences for $G\alpha_{i1/2}$ ($F_{10,39} = 4.44$, $P < 0.0001$) but not for $G\alpha_{i3}$ ($F_{10,34} = 0.61$, $P = 0.79$) and $G\alpha_{o}$ ($F_{10,33} = 0.68$, $P = 0.73$) levels after EEDQ.

receptors at steady state, a state to which the system tends to return after irreversible inactivation of the receptors. To assess the experimental uncertainty of this parameter (r/k) for further statistical comparisons, the experimental data were also fitted to the equation:

$$R_t = R_{ss}(1 - e^{-kt}) \quad (2)$$

were R_{ss} corresponds to the r/k value, a constant that has to be estimated.

Because the recovery of [^{3}H]UK 14304 agonist binding sites after EEDQ showed a marked deviation (biphasic aspect) from the predicted monoexponential curve (see Fig. 3A), turnover data (all individual experiments together) were also analyzed according to more complex models. First, recovery data were fitted to a biexponential model defined by the equation:

$$R_t = r_1/k_1(1 - e^{-k_1 t}) + r_2/k_2(1 - e^{-k_2 t}). \quad (3)$$

This model, which assumes the existence of two different populations of receptors, resulted in a better fit than that obtained with the monoexponential model ($F_{2,47} = 9.07$; $P < 0.0005$). However, turnover parameters with this model were estimated with very large standard errors (data not shown). Moreover, this model (Eq. (3)) continued to show a marked deviation from the experimental recovery data. Finally, recovery data were empirically analyzed according to a biphasic model which resulted in the best fit. The proposed new model has two phases for the recovery of agonist binding sites with different turnover rates for each phase. Therefore, recovery data for the biphasic model were fitted to the equation:

$$R_t = \text{if} \begin{cases} t < T; r_1/k_1(1 - e^{-k_1 t}) \\ t \geq T; r_1/k_1(1 - e^{-k_1 T}) + r_2/k_2(1 - e^{-k_2(t-T)}) \end{cases} \quad (4)$$

where the first phase of recovery of [^{3}H]UK 14304 binding sites was defined by r_1 and k_1, and the second phase of recovery, which begins at a time T after EEDQ, was defined by the turnover parameters r_2 and k_2 (compare with Eqs. (1) and (3)). This biphasic model resulted in a better fit than that obtained with the biexponential model ($F_{1,46} = 5.88$; $P < 0.02$). Thus, the biphasic model showed an excellent agreement with the experimental turnover data (see Fig. 3A).

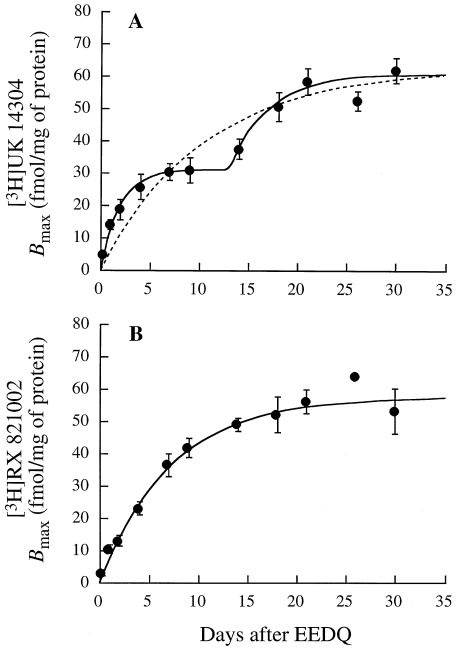

Fig. 3. Recovery of α_2-adrenoceptor density in the rat cerebral cortex after EEDQ-induced receptor inactivation. Rats were killed 6 h and 1, 2, 4, 7, 9, 14, 18, 21, 26 and 30 days after the administration of EEDQ (1.6 mg kg^{-1}, i.p.). The B_{max} values were determined from complete saturation experiments for the agonist [^{3}H]UK 14304 (A) and for the antagonist [^{3}H]RX 821002 (B) (6×10^{-11} to 8×10^{-9} M), using the non-linear regression program LIGAND. Data shown are means ± S.E.M. derived from three to five experiments. Range for K_d values during recovery was 0.9 ± 0.1 to 1.2 ± 0.1 nM for [^{3}H]UK 14304 and 0.4 ± 0.1 to 0.8 ± 0.2 nM for [^{3}H]RX 821002 ($F_{10,40} = 0.55$; $P = 0.89$ and $F_{10,18} = 0.90$; $P = 0.55$, respectively). The solid lines represent the computer-assisted curve fitting of experimental data to the best model (i.e., the biphasic model described by Eq. (4) for the recovery of [^{3}H]UK 14304 binding sites, and the monoexponential model described by Eq. (1) for the recovery of [^{3}H]RX 821002 binding sites). The dashed line represents the computer-assisted curve fitting of experimental recovery data of [^{3}H]UK 14304 binding sites to the monoexponential model. See Tables 1 and 2 for other details and turnover parameters.

2.6. Statistics

Immunoblot and radioligand binding data are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Scheffé's test was used for the statistical evaluations. The level of significance was chosen as $P = 0.05$. Receptor turnover parameters are expressed as the best fit values ± S.E. determined by the matrix inversion method, using the non-linear regression program GraFit (Leatherbarrow, 1990). Standard error values determined by non-linear regression were not used in further formal statistical calculations. Comparisons of experimental data sets for the recovery of α_2-adrenoceptor density were performed by comparing the goodness-of-fit of a model with and without a set of constraints by means of a *F*-test. The selection between the different recovery models was made statistically, using the extra sum of squares principle (*F*-test) as outlined by Munson and Rodbard (1980). The more complex model was accepted if the *P*-value resulting from the *F*-test was less than 0.05. For further details see the work of Barturen and García-Sevilla (1992) and other references therein.

2.7. Materials

[^{3}H]UK 14304 (bromoxidine, specific activity, 60–87 Ci mmol^{-1}) was purchased from Du Pont/New England Nuclear (Schwalbach/Tannus, Germany) and [^{3}H]RX 821002 (2-methoxy idazoxan, specific activity, 48–57 Ci mmol^{-1}) was purchased from Amersham International (Bucks, UK). Rabbit anti-G protein subunits polyclonal antisera raised against specific C-terminal peptides or the N-terminus ($G\alpha_o$) were purchased from Dupont/New England Nuclear: anti-$G\alpha_{i1/2}$ (AS/7), anti-$G\alpha_{i3}$ (EC/2) and anti-$G\alpha_o$ (GC/2). Horseradish peroxidase-labelled donkey antirabbit immunoglobulin G and Enhanced Chemiluminescence reagents were obtained from Amersham International. Hyperfilm ECL film from Amersham International was used for autoradiography. Other drugs (and their sources) included: (−)-adrenaline bitartrate, EEDQ and 5-HT (Sigma, USA); 2-[2-[4-(*o*-methoxyphenyl)piperazine-1-yl]ethyl]-4,4-dimethyl-1,3(2*H*,4*H*)-isoquinolinedione HCl (ARC 239) (Thomae, Biberach, Germany); 2-[2*H*-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole HCl (BRL 44408) (SmithKline Beecham, Essex, UK). Other reagents were obtained from Sigma.

3. Results

3.1. Effects of EEDQ on regulatory G_i protein subunits

A single dose of EEDQ (1.6 mg kg^{-1}, i.p.), which has been reported to almost completely inactivate cortical α_2-adrenoceptors (Ribas et al., 1993; see also Fig. 3), induced a rapid increase in the immunoreactivity of cortical $G\alpha_{i1/2}$ proteins (30% at 6 h, $P < 0.05$). This reached a maximum at 4 days (45%, $P < 0.05$) and then slowly returned (7–30 days) to control values (Figs. 1 and 2). In contrast, the levels of $G\alpha_{i3}$ and $G\alpha_o$ immunoreactivity after EEDQ (6 h–30 days) did not change significantly (Figs. 1 and 2).

3.2. Differential recovery of agonist and antagonist binding sites after irreversible inactivation by EEDQ

Treatment with EEDQ (1.6 mg kg^{-1}, i.p., for 6 h) induced an almost complete loss of α_2-adrenoceptors from the cerebral cortex (B_{max} for the agonist [^{3}H]UK 14304 and the antagonist [^{3}H]RX 821002 reduced by > 90%), followed by a progressive recovery of receptor density (1–30 days). These experiments provided the B_{max} values for the analysis of the exponential recovery functions (Fig. 3), whose parameters are summarized in Table 1. The simultaneous analysis of recovery data with [^{3}H]UK 14304 and [^{3}H]RX 821002 according to a sole monoexponential function (same *r* and *k* values) did not differ from the analysis without constraints (i.e., independent *r* and *k* values for each radioligand), which suggested very similar agonist and antagonist turnover functions (Table 1).

Table 1
Monoexponential turnover parameters of brain α_2-adrenoceptors labelled with the agonist [^{3}H]UK 14304 and the antagonist [^{3}H]RX 821002

Radioligand	Turnover parameters			
	r (fmol mg protein^{-1} day^{-1})	*k* (day^{-1})	$t_{1/2}$ (days)	*r*/*k* (fmol mg protein^{-1})
[^{3}H]UK 14304	5.8 ± 0.6	0.094 ± 0.016	7.4 ± 1.3	62 ± 4
[^{3}H]RX 821002	8.1 ± 0.8	0.140 ± 0.018	4.9 + 0.7	57 ± 3

Rats were injected with a single dose of the irreversible antagonist EEDQ (1.6 mg kg^{-1}, i.p.) and killed after different periods of time to assess the recovery of the specific binding of [^{3}H]UK 14304 or [^{3}H]RX 821002 to cortical membranes. Receptor turnover parameters were calculated from data shown in Fig. 3 (simultaneous analysis of three to five experiments). Reappearance of receptors was assessed by non-linear analysis according to the equation $R_t = (r/k)(1 - e^{-kt})$, where *r* is the rate constant for the appearance of receptors, *k* is the rate constant for the disappearance of receptors and the ratio *r*/*k* is the density of receptors at steady state after irreversible inactivation; $t_{1/2}$ represents the apparent half-life of the receptor and was calculated from the equation: $t_{1/2} = \ln 2/k$. Turnover parameters are expressed as the best fit values ± S.E. calculated by the matrix inversion method, using the non-linear regression program GraFit (Leatherbarrow, 1990). Data analysis according to a sole monoexponential function (same *r* and *k* values) compared with a model without constraints (two different *r* and *k* values) did not detect significant differences between turnover parameters for agonist-labelled and antagonist-labelled α_2-adrenoceptors ($F_{2,76} = 0.73$; $P = 0.48$).

Table 2
Biphasic turnover parameters of brain α_2-adrenoceptors labelled with the agonist [^{3}H]UK 14304

Assay conditions	Turnover parameters								
	r_1 (fmol mg protein^{-1} day^{-1})	k_1 (day^{-1})	$(t_{1/2})_1$ (days)	r_1/k_1 (fmol mg^{-1} protein)	r_2 (fmol mg protein^{-1} day^{-1})	k_2 (day^{-1})	$(t_{1/2})_2$ (days)	r_2/k_2 (fmol mg^{-1} protein)	T (days)
$+MnCl_2$	15.7±3.4	0.513±0.136	1.4±0.4	31±2	7.4±3.0	0.254±0.121	2.7±1.7	29±4	13±1
$-MnCl_2$	13.0±3.3	0.555±0.166	1.3±0.4	23±3	3.8±4.0	0.204±0.244	3.4±4.1	19±5	10±5

Receptor turnover parameters were calculated from simultaneous analysis of three to five experiments. Other details as for Table 1. Experimental data for the recovery of [^{3}H]UK 14304 binding (B_{max}) to rat cortical membranes after EEDQ were determined from complete saturation experiments performed in the presence (Fig. 3A) or absence (receptor recovery not shown) of 0.1 mM $MnCl_2$ in the incubation medium. Reappearance of [^{3}H]UK 14304 binding sites was assessed by non-linear analysis according to the biphasic equation R_t = if $t < T$ then $(r_1/k_1)(1-e^{-k_1 t})$; if $t \geq T$ then $(r_1/k_1)(1-e^{-k_1 T})+(r_2/k_2)(1-e^{-k_2(t-T)})$ where R_t represents the density (B_{max}, in fmol mg protein^{-1}) of [^{3}H]UK 14304 binding at a given discrete time t (days), r_1 and r_2 are the rate constants of appearance and k_1 and k_2 are the rate constants of disappearance of the receptors before and/or after a time T. Turnover parameters are expressed as the best fit values ± S.E. calculated by the matrix inversion method, using the non-linear regression program GraFit (Leatherbarrow, 1990). Computer-assisted curve fitting demonstrated that in the presence or absence of $MnCl_2$ the proposed biphasic model (Eq. (4)) was significantly better than the monoexponential model (Eq. (1)) ($F_{3,46} = 7.17$, $P = 0.0005$ and $F_{3,28} = 3.91$, $P = 0.019$, respectively). Data analysis according to a sole biphasic function (same r_1, k_1, r_2, k_2 and T values) resulted in a significant decrease of goodness of fit compared with that of a model without constraints (i.e., different turnover parameters for each recovery) ($F_{5,74} = 9.28$, $P < 0.0001$).

However, the recovery of [^{3}H]UK 14304 agonist binding sites after EEDQ showed a marked deviation from the predicted monoexponential curve (Fig. 3A). The biphasic nature of the recovery data could reflect non-fulfilment of one of the implicit assumptions for the monoexponential model, i.e., that the rate of receptor appearance is constant during the repopulation period. In order to investigate the relevance of this observation, an alternative biphasic recovery model was used (see Eq. (4)).

With the proposed biphasic model (Eq. (4)), the analysis of recovery of [^{3}H]UK 14304 binding sites in the rat cerebral cortex after EEDQ resulted in a better fit than that obtained with the monoexponential model (Eq. (1)) (Fig. 3, Table 2) or the biexponential model (Eq. (3)) (data not shown). The recovery of [^{3}H]UK 14304 binding, measured without the addition of $MnCl_2$ in the incubation medium, resulted, as expected, in lower B_{max} values for agonist binding sites. Under these conditions, the best fit of data was also to the biphasic model (Table 2). According to this model, the first phase of recovery would correspond to a rapid turnover of agonist binding sites with higher r_1 and k_1 rate constants, and a half-life of about 1.5 days. The limit of the recovery function (r_1/k_1) for the first phase was approximately one-half of the total density at steady state (Table 2). The second phase of recovery was estimated to start 10–13 days (T) after the first phase and would correspond to a slower turnover of agonist binding sites (Table 2). In marked contrast, the proposed biphasic model did not fit the recovery data for [^{3}H]RX 821002 antagonist binding sites after EEDQ (Fig. 3B).

3.3. Recovery of α_2-adrenoceptor subtypes after EEDQ-induced receptor inactivation

Because the agonist [^{3}H]UK 14304 has different affinity for the various α_2-adrenoceptor subtypes, the biphasic nature of its recovery after EEDQ could be related to a differential recovery of receptor subtypes. Therefore, competition experiments with [^{3}H]RX 821002 (non-selective α_2-adrenoceptor antagonist) and subtype-specific antagonists were performed to delineate the recovery of receptor subtypes after EEDQ.

Competition experiments with BRL 44408 (85-fold α_{2A} selective relative to $\alpha_{2B/C}$) and ARC 239 (100-fold $\alpha_{2B/C}$ selective relative to α_{2A}) were carried out with control rats and the results were compared with those obtained at 7 and 21 days after EEDQ (i.e., the best time intervals defining the plateaux of the biphasic recovery, Fig. 3). In control rats, as well as in rats treated with EEDQ for 7 and 21 days, the inhibition curves for both antagonists were clearly monophasic and the best fits were to single populations of binding sites with high affinity for BRL 44408 (control: $K_i = 24$ nM, $B_{max} = 101$ fmol mg^{-1} of protein; EEDQ: $K_i = 25$ and 23 nM, $B_{max} = 52$ and 86 fmol mg^{-1} of protein; $n = 3$) and low affinity for ARC 239 (control: $K_i = 0.81$ μM, $B_{max} = 98$ fmol mg^{-1} of protein; EEDQ: $K_i = 0.72$ and 0.76 μM, $B_{max} = 51$ and 83 fmol mg^{-1} of protein; $n = 3$), which indicated a predominance of the α_{2A}-subtype in the cerebral cortex. The very similar K_i values for BRL 44408 and ARC 239 in control and EEDQ-treated (7 and 21 days) rats clearly indicated that the populations of receptor subtypes (α_{2A} vs. $\alpha_{2B/C}$) did not change during the recovery of α_2-adrenoceptors. Therefore, the biphasic nature of recovery of [^{3}H]UK 14304 binding sites after EEDQ (Fig. 3) cannot be explained by a differential recovery of α_2-adrenoceptor subtypes.

4. Discussion

The quantitative evaluation of receptor repopulation after irreversible inactivation has proved to be a powerful method to assess the turnover of adrenoceptors (Mahan et al., 1987) and its pharmacologic modulation (Barturen and García-Sevilla, 1992; Ribas et al., 1993; Gabilondo and García-Sevilla, 1995). In the present in vivo study, a low dose of the peptide-coupling agent EEDQ (1.6 mg kg^{-1}) was used as the irreversible antagonist for brain α_2-adrenoceptors (Adler et al., 1985; Pilc et al., 1992; Miralles et al., 1993b). The dose of EEDQ used excluded confounding factors related to the alkylation of other receptors (i.e., possible altered cross-talk between receptors) because much higher doses of EEDQ (6–10 mg kg^{-1}) are needed to elicit substantial irreversible inactivation of other neurotransmitter receptors (Meller et al., 1985, 1988), and this makes receptor turnover studies with this tool possible (Leff et al., 1984; Lévesque and Di Paolo, 1991; Nowak and Zak, 1991; Pinto and Battaglia, 1994). Because EEDQ does not discriminate between α_2-adrenoceptor subtypes (Barturen and García-Sevilla, 1992) and the radioligands [^{3}H]UK 14304 and [^{3}H]RX 821002 are mixed $\alpha_{2A/B/C}$-adrenoceptor agents (Sastre and García-Sevilla, 1994), the receptors measured are simply termed α_2-adrenoceptors. However, the predominant α_2-adrenoceptor in the rat cerebral cortex is the orthologue $\alpha_{2A/D}$-subtype (Barturen and García-Sevilla, 1992; Nicholas et al., 1993 and present results). Furthermore, the α_{2A}-subtype appears to mediate many of the relevant effects of α_2-adrenoceptor agonists (MacDonald et al., 1997).

Turnover functions for [^{3}H]labelled agonist and [^{3}H]labelled antagonist α_2-adrenoceptors, analyzed (Eq. (1)) under the same conditions (i.e., absence of Mn^{2+} in the assay), suggested that the rate of receptor appearance (r) obtained by using an agonist radioligand may underestimate the true r value for the receptor population. Thus, the r value obtained with [^{3}H]UK 14304 was lower (36%) than that obtained with [^{3}H]RX 821002, which is consistent with the reduced density of [^{3}H]UK 14304 binding sites (B_{max} 35% lower) compared with that of [^{3}H]RX 821002 binding sites (data not shown). These results were

similar to those reported in an in vitro study of α_2-adrenoceptor turnover in which the adenocarcinoma cell line HT29 and the agonist [^{3}H]UK 14304 and the antagonist [^{3}H]yohimbine (r and B_{max} values for the agonist were 35 and 33% lower, respectively) were used (Paris et al., 1987). Similar results have been reported for agonist- and antagonist-labelled 5-HT_{2A} receptors in the rat cerebral cortex (Pinto and Battaglia, 1994). In contrast, turnover parameters for agonist-labelled α_2-adrenoceptors, with Mn^{2+} in the assay, did not differ significantly from those of antagonist-labelled α_2-adrenoceptors. This indicated the importance of Mn^{2+}, which enhances maximally the labelling of agonist binding sites, for accurate assessment of turnover parameters when using agonist radioligands.

In the present study, an alternative recovery model (Eq. (4)) was proposed which, regardless of the presence of Mn^{2+} in the assay, clearly discriminated between turnover functions for agonist- and antagonist-labelled α_2-adrenoceptors. Thus, recovery of binding sites for [^{3}H]UK 14304, but not for [^{3}H]RX 821002, fitted best to the proposed biphasic model. This model suggested the existence of two phases of recovery for agonist binding sites with different turnover rates. Crucial to the proposed biphasic model is the period of time allowed for receptor recovery after EEDQ (30 days in the present study) in order to reach the steady state density of agonist binding sites before blockade and to have enough data for non-linear analysis and statistical evaluation. Previous studies may have missed the biphasic nature of recovery of agonist binding sites because shorter periods of time were allowed for receptor recovery after EEDQ (7–16 days in most studies) (Adler et al., 1985; Barturen and García-Sevilla, 1992; Durcan et al., 1994; Gabilondo and García-Sevilla, 1995) (see Fig. 1). However, the proposed biphasic model of recovery of agonist binding sites provides a crude approximation of this complex phenomenon and should be validated with new experimental designs (e.g., agonist turnover function after drug-induced receptor down-regulation).

The possibility that the biphasic recovery of agonist binding sites after EEDQ could be related to a sequential recovery of different α_2-adrenoceptor subtypes was discarded. Competition experiments with [^{3}H]RX 821002 (mixed $\alpha_{2A/B/C}$ antagonist) (Uhlén and Wikberg, 1991; Hudson et al., 1992; Uhlén et al., 1992) and subtype-selective antagonists such as BRL 44408 (85-fold α_{2A} selective relative to $\alpha_{2B/C}$) (Young et al., 1989; Sastre and García-Sevilla, 1994) and ARC 239 (100-fold $\alpha_{2B/C}$ selective relative to α_{2A}) (Bylund et al., 1988; Sastre and García-Sevilla, 1994) clearly indicated that the affinity and relative proportion of receptor subtypes did not change during the first and second phase of recovery of α_2-adrenoceptors.

In the proposed biphasic model, the recovery of agonist binding sites starts with a first phase of rapid turnover, which is followed (10–13 days later) by a second phase with a slower turnover. In the mouse brain, the half-life of α_2-adrenoceptors, estimated from recovery studies after EEDQ (2 mg kg^{-1}, i.p.) and with [^{3}H]RX 821002, is 5.25 days (Durcan et al., 1994), very similar to that reported in the present study ($t_{1/2} = 4.9$ days) and similar to that reported for [^{3}H]idazoxan ($t_{1/2} = 4.1$ days) in rat brain (Adler et al., 1985). Moreover, Durcan et al. (1994) reported that medetomidine (α_2-adrenoceptor agonist)-induced sedation and hypothermia returned to control values by 8 and 12 days, respectively, after EEDQ administration, which is coincident with the plateau of the first phase for the recovery of agonist binding sites (Fig. 1A). Also, the half-life corresponding to the first phase of [^{3}H]UK 14304 binding site recovery after EEDQ ($t_{1/2} = 1.3–1.4$ days) was very similar to that reported ($t_{1/2} = 2.4$ days (Adler et al., 1985) and $t_{1/2} = 1.28$ days (Agneter et al., 1993)) for the recovery of the noradrenaline release-inhibiting effects of UK 14304 on rat cortical α_2-autoreceptors after EEDQ.

Because functional recovery is more rapid than the recovery of the receptor pool, it has been suggested that there is a large receptor reserve for α_2-adrenoceptors (Adler et al., 1985; Agneter et al., 1993; Durcan et al., 1994). The good relation between the first phase of the recovery of agonist binding sites and the restoration of functional responses of α_2-adrenoceptors after irreversible inactivation by EEDQ could suggest, therefore, that the second phase of the recovery of agonist binding sites may be that of the receptor reserve. In this sense, it is noteworthy that the rate constant for the appearance of receptor in the second phase of the recovery of agonist binding sites was very similar to that for the recovery of antagonist binding sites (7.4 and 8.1 fmol mg protein^{-1} day^{-1}, respectively).

It is currently accepted that the rate of receptor appearance (r) reflects the summed rates of transcriptional, translational, and post-translational processes, but when using [^{3}H]labelled agonists in receptor turnover studies another factor should be taken into account, i.e., the coupling between receptor and G protein subunits to form the high-affinity state labelled by the agonist (Kenakin, 1997). In this context, a possible explanation for the proposed biphasic recovery model emerged from the effects of EEDQ on $G\alpha_{i1/2}$ proteins. The present results suggest that the biphasic nature of recovery of agonist-labelled α_2-adrenoceptors could be related to the rapid over-expression and time course of $G\alpha_{i1/2}$ proteins after EEDQ (i.e., rapid up-regulation of the preferred coupling G protein as an adaptive mechanism after in vivo receptor alkylation). Conversely, sustained stimulation of α_2-adrenoceptors by the agonist UK 14304 in rat 1 fibroblast cells has been shown to result in a marked down-regulation of $G\alpha_{i2}$ proteins (Grassie and Milligan, 1995). At present, however, there is no direct evidence for a causal relation between the over-expression of $G\alpha_{i1/2}$ proteins and the biphasic nature of recovery of agonist binding sites in this model. Further studies are needed to unravel the possible relevance of this phenomenon.

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