Title: α1-adrenoceptors in the rat cerebral cortex: new insights into the characterization of α1L- and α1D-adrenoceptors

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Abstract: Among the three α1-adrenoceptor subtypes (α1A, α1B and α1D) a peculiar intracellular localization and poor coupling to membrane signals of cloned α1D-adrenoceptor has been reported. In addition, the α1L-adrenoceptor (low affinity for prazosin), a functional phenotype of α1A, has been described. The purpose of this work was to analyze the expression, cellular localization and coupling to membrane signalling (inositol phosphate accumulation) of α1-adrenoceptor subtypes in a native tissue, the rat cerebral cortex. mRNA for the three subtypes was quantified by real-time RT-PCR (α1D>α1B>>α1A). α1-adrenoceptors were also detected by immunoblotting, revealing α1A- and α1B-adrenoceptors to be predominantly expressed in the membrane fraction and the α1D-adrenoceptor to be localized in the cytosolic fraction. Competitive radioligand binding studies revealed the presence of α1D-adrenoceptor in tissue homogenates, whereas only α1A- and α1B-subtypes were detected in membranes. The proportion of α1A-adrenoceptor increased after treatment with noradrenaline, suggesting differences in agonist-mediated trafficking. Saturation experiments detected high- and low (α1A/L)-prazosin binding sites, the latter of which disappeared on incubation with GppNHp. The α1A/L-adrenoceptor was heavily implicated in the inositol phosphate response, while the α1D-subtype did not play a relevant role. These results suggest that the predominant cytosolic localization of α1D-adrenoceptor lies behind its poor coupling to membrane signalling such as inositol phosphate pathway. The fact that the α1L-adrenoceptor detected in radioligand binding studies disappeared in the presence of GppNHp implies that it represents a conformational state of the α1A-adrenoceptor coupled to G protein.
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α₁-adrenoceptors in the rat cerebral cortex: new insights into the characterization of α₁L- and α₁D-adrenoceptors

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Abstract
Among the three $\alpha_1$-adrenocceptor subtypes ($\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$) a peculiar intracellular localization and poor coupling to membrane signals of cloned $\alpha_{1D}$-adrenocceptor has been reported. In addition, the $\alpha_{1L}$-adrenocceptor (low affinity for prazosin), a functional phenotype of $\alpha_{1A}$, has been described. The purpose of this work was to analyze the expression, cellular localization and coupling to membrane signalling (inositol phosphate accumulation) of $\alpha_1$-adrenocceptor subtypes in a native tissue, the rat cerebral cortex. mRNA for the three subtypes was quantified by real-time RT-PCR ($\alpha_{1D}>\alpha_{1B}>\alpha_{1A}$). $\alpha_1$-adrenocceptors were also detected by immunoblotting, revealing $\alpha_{1A}$- and $\alpha_{1B}$-adrenocaptors to be predominantly expressed in the membrane fraction and the $\alpha_{1D}$-adrenocceptor to be localized in the cytosolic fraction. Competitive radioligand binding studies revealed the presence of $\alpha_{1D}$-adrenocceptor in tissue homogenates, whereas only $\alpha_{1A}$- and $\alpha_{1B}$-subtypes were detected in membranes. The proportion of $\alpha_{1A}$-adrenocceptor increased after treatment with noradrenaline, suggesting differences in agonist-mediated trafficking. Saturation experiments detected high- and low ($\alpha_{1A/L}$)-prazosin binding sites, the latter of which disappeared on incubation with GppNHp. The $\alpha_{1A/L}$-adrenocceptor was heavily implicated in the inositol phosphate response, while the $\alpha_{1D}$-subtype did not play a relevant role. These results suggest that the predominant cytosolic localization of $\alpha_{1D}$-adrenocceptor lies behind its poor coupling to membrane signalling such as inositol phosphate pathway. The fact that the $\alpha_{1L}$-adrenocceptor detected in radioligand binding studies disappeared in the presence of GppNHp implies that it represents a conformational state of the $\alpha_{1A}$-adrenocceptor coupled to G protein.
Keywords: $\alpha_1$-adrenoceptor subtypes; rat cerebral cortex; $\alpha_{1D}$-intracellular localization; $\alpha_{1L}$-adrenoceptors; [$^3$H]prazosin binding studies; G-proteins.
1. Introduction

Pharmacological and molecular cloning studies have confirmed the existence of three $\alpha_1$-adrenoceptors subtypes: $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$ (Alexander et al., 2008) that have been distinguished by their affinity for different compounds in radioligand binding and functional studies. They also differ in their subcellular distribution: $\alpha_{1B}$-adrenoceptors are expressed predominantly in the plasma membrane (Chalothorn et al., 2002; Hirasawa et al., 1997; McCune et al., 2000), $\alpha_{1D}$-adrenoceptors are localized mainly in intracellular compartments (Chalothorn et al., 2002; McCune et al., 2000), and the $\alpha_{1A}$-subtype is present both on the cell surface and in intracellular pools (Chalothorn et al., 2002; Hirasawa et al., 1997).

We hypothesized that the cytosolic localization of $\alpha_{1D}$-adrenoceptors may explain the difficulty of detecting this subtype by radioligand binding in membrane preparations from native tissues expressing significant $\alpha_{1D}$-adrenoceptor mRNA levels (Yang et al., 1997). We suspected that this peculiar expression pattern may also explain the poor coupling of $\alpha_{1D}$-adrenoceptors to membrane signals such as inositol phosphate accumulation (Schwinn et al., 1995; Theroux et al., 1996). The present work tests these hypotheses.

Additionally, the existence of a fourth $\alpha_1$-adrenoceptor, the $\alpha_{1L}$-adrenoceptor, which shows a relatively low affinity for prazosin (pKi<9), has been justified mainly by functional data. The response induced by $\alpha_1$-adrenoceptor stimulation in several tissues is mediated by the $\alpha_{1L}$-subtype. This is the case of the human, rat, guinea-pig and rabbit prostate (Ford et al., 1996; Hiraoka et al., 1999; Pennefather et al., 1999; van der Graaf et al., 1997), rat and human vas deferens (Amobi et al., 2002; Ohmura et al., 1992), rabbit iris (Suzuki et al., 2002), rat small mesenteric arteries (Stam et al., 1999), canine
subcutaneous resistance arteries (Argyle and McGrath, 2000) and rabbit ear artery
(Hiraizumi-Hiraoka et al., 2004). However, no gene encoding the $\alpha_{1L}$-adrenoceptor has
been identified to date, and it has been proposed that this subtype represents a particular
conformational state of the cloned $\alpha_{1A}$-adrenoceptor (Ford et al., 1997; Marti et al.,
2005). This has been confirmed by recent studies using $\alpha_{1A}$-adrenoceptor “knockout”
mice, which have shown that the $\alpha_{1L}$-adrenoceptor phenotype is observed only when the
$\alpha_{1A}$-adrenoceptor gene is present (Gray et al., 2008; Muramatsu et al., 2008). However,
the mechanism involved in the manifestation of $\alpha_{1L}$-phenotype pharmacology is yet to
be established.

In the present study we characterized the $\alpha_{1D}$- and $\alpha_{1A/L}$-adrenoceptors in native
tissue. We chose the rat cerebral cortex, which possesses the mRNA for the three $\alpha_{1}$-
adrenoceptors (Day et al., 1997). We analyzed the coupling of these receptors to Gq
proteins in the cell membrane by determining the inositol phosphate accumulation
mediated by each subtype. The mRNA of $\alpha_{1}$-adrenoceptors was quantified by real-time
RT-PCR. In addition, the protein expression that was determined by radioligand binding
and immunoblotting was assessed in membranes, cytosolic and crude extracts.
Considering that the $\alpha_{1L}$ subtype has been detected mainly in functional studies, we
hypothesized that this conformational state of the $\alpha_{1A}$-adrenoceptor would be modulated
by guanine nucleotide. Thus, saturation radioligand binding experiments were
performed in the presence or absence of a non-hydrolyzable analogue of GTP
(GppNHp:guanosine-5’-(\(\beta, \gamma\)-imido)triphosphate)).
2. Materials and methods

2.1. Animals

Female Wistar rats (200-220 g) bred in our faculty’s animal facility were housed under a 12-h light/dark cycle at 22°C and 60% humidity. They were anaesthetized with Isoflurane prior to sacrifice. All experimental procedures complied with institutional guidelines and were approved by the Experimental Animal Ethics Committee of the Faculty of Pharmacy of the University of Valencia (Spain).

2.2. Accumulation of [3H]-inositol phosphates

The determination of total inositol phosphate accumulation was adapted from the method used by Berridge et al. (1982). The rats’ brains were quickly removed from their skulls and the cortex of both hemispheres was sliced (350 μm cubes) on a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY, USA) and then pooled and incubated at 37 ºC for 30 min in physiological solution (composition in mM: NaCl 118, KCl 4.7, CaCl2 1.8, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, and glucose 11, aerated with 95% O2 / 5% CO2). The solution was changed twice. Subsequently, the tissues were labelled for 2 h at 37 ºC in 10 μCi ml⁻¹ of myo-[3H]inositol in physiological solution. The mixture was vigorous shaked during the whole period and was aerated every 15 min. Following incubation, samples were washed three times with physiological solution. The cerebral cortex slices were allowed to settle with gravity, after which the buffer was removed by aspiration. To measure the production of inositol phosphates, 50 μl of packed cortex slices (2 mg of protein) were incubated at 37 ºC in an atmosphere of 95% O2 and 5% CO2 in a final volume of 250 μl of physiological solution. LiCl (10 mM) was added to the solution in order to inhibit the
metabolism of inositol monophosphates. The tissues were incubated for 30 min with increasing concentrations of noradrenaline (0.1 \( \mu M \)–1 mM).

To determine the antagonist action of prazosin (0.1 nM-100 \( \mu M \)), 5-methylurapidil (0.1 nM-100 \( \mu M \)) and BMY 7378 (0.1\( \mu M \)-100 \( \mu M \)) on noradrenaline–induced inositol phosphate accumulation, the tissues were incubated for 30 min with different concentrations of the antagonist, and were subsequently stimulated for 30 min with a maximal concentration of noradrenaline (100 \( \mu M \)). Unstimulated (basal) and noradrenaline-stimulated (maximal response) inositol phosphate accumulation were simultaneously determined. Each experimental value was performed in triplicate. Incubation was terminated by placing the samples in a cold water bath (4\(^\circ\)C) and adding 2 ml of a cold mixture of methanol/chloroform/HCl (40:20:1, v/v/v). The samples were sonicated for 35 min at 2–3\(^\circ\)C and, following addition of 0.63 ml of chloroform and 1.26 ml of distilled water, were centrifuged at 2500 \( g \) for 10 min to facilitate phase separation. The aqueous layer was removed from the tubes to assay the inositol phosphate formation. Each sample was neutralized and run through an AG1-X8 column, formate form, 100-200 mesh (Bio-Rad, Hercules, CA). The resin was washed successively with 6 ml of water and 6 ml of 60 mM ammonium formate-5 mM sodium tetraborate to eliminate free myo-[\(^3\)H]inositol and glycerophosphoinositol, respectively. Total inositol phosphates were eluted with 3 ml of 1 M ammonium formate-0.1 M formic acid. The eluent fractions were collected and counted in a scintillation counter.

The accumulation of \([\(^3\)H]\)inositol phosphates was calculated as the percentage (dpm%) of \([\(^3\)H]\)inositol-labeled lipids in each sample to account for interexperimental variations in labelling and sample sizes, and was expressed as percentages of basal \([\(^3\)H]\)inositol phosphate accumulation. Inositol phosphate accumulation in the presence
of the different concentrations of antagonist employed was expressed as a percentage of the maximum increase obtained in the presence of the agonist after subtracting the basal inositol phosphate accumulation.

Concentration-response data for both noradrenaline-induced [3H] inositol phosphate accumulation and antagonist inhibition were fit to non-linear regression plot using Prism version 4.0 (GraphPad Software; San Diego, California, U.S.A). The data were fitted to mono or biphasic sigmoidal functions and the best fit to one-site or two-site models was evaluated using the F-test (P<0.05). Affinity values of antagonists (pK_b) were calculated according to the equation by Leff and Dougall (1993) [K_b= IC_50/(2+[A]/EC_50)^nH]^{1/nH}-1), where [A] is the concentration of agonist (100 μM noradrenaline) used and n_H the curve slope.

2.3. Real-time quantitative RT-PCR

The cerebral cortex was dissected and rapidly frozen in liquid nitrogen and then powdered in a mortar and dissolved in TriPure Isolation Reagent (Roche). Total RNA was obtained from the cerebral cortex following the manufacturer’s instructions. Rat genomic DNA, used as a standard in real-time PCR, was isolated from a crude nuclear fraction of liver, as previously described in Marti et al. (2005). The sequences of the oligonucleotide primers used in this study, their positions in the corresponding mRNA sequences and the expected sizes for the PCR products are shown in Table 1.

Quantitative analysis of the mRNAs encoding the three α1-adrenoceptor subtypes was performed by real-time RT-PCR with a Gene Amp 5700 sequence detection system (Applied Biosystems). The PCR reaction took place over 40 cycles and consisted of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 20 s.
The threshold cycle values (Ct) obtained for each α1-adrenoceptor subtype in each reverse transcription reaction were interpolated in the standard plots generated with the genomic DNA, and the copy number per µg of RNA were calculated using the Gene Amp 5700 sequence detection system software (Applied Biosystems). These absolute values were normalized with the copy number values obtained for Gapdh (Glyceraldehyde-3-phosphate dehydrogenase).

2.4. Western Blot experiments

To obtain membrane and cytosolic proteins, the frozen cerebral cortex was ground to a powder in a mortar and homogenized using an Ultra-Turrax T25 dispersing instrument (IKA® Jankel & Kunkel, Staufen, Germany) in 5 mM of Tris-HCl buffer (pH=7.5) containing 250 mM sucrose, 1mM EDTA (ethylenediaminotetraacetic acid) and protease inhibitor cocktail (Complete®, Roche Applied Science, Germany). They were then centrifuged at 13,000 g for 15 min at 4ºC and ultra-centrifuged at 80,000 g for 60 min at 4ºC. The supernatant containing cytosolic proteins was stored at –80ºC until required. Pellets containing the membrane fraction were lysed in ice-cooled lysis buffer (0.1% sodium dodecyl sulphate, 1% Triton, 1% sodium deoxycolate) containing protease inhibitor cocktail (Complete®, Roche Applied Science, Germany) and were then centrifuged at 16,000 g for 15 min at 4ºC. The supernatant containing membrane proteins was stored at –80ºC until required. The protein concentration was determined by the Bradford method (1976) (BioRad Laboratories, Inc.).

Western blots of the different protein extracts (150 µg) were performed following the method developed by Laemmli (1970) and the modifications made by Oliver et al. (2009), using goat polyclonal antibody (Santa Cruz Biotechnology) against
α₁A-adrenoceptor (sc-1477, 1:100), α₁B-adrenoceptor (sc-1476, 1:100) and α₁D-adrenoceptor (sc-1475, 1:250) and rabbit Anti-Actin (A2066 1:2000, Sigma-Aldrich) as a loading control. After incubation with ECL® western blotting detection reagent (Amershan Bioscience), bands were immediately documented with an Autochemi™ BioImaging System using Labworks 4.6 capture software (Ultra-Violet Products Ltd., Cambridge, UK). Quantification of the western blotting experiments was performed by optic densitometry, and the integrated optic density value was calculated. Results were normalized and expressed as the ratio to actin.

2.5. Radioligand binding studies

2.5.1. Tissue preparation

Two different preparations (crude tissue homogenates and tissue membranes) were obtained from the rat cerebral cortex, which was dissected, rapidly frozen and stored at -80°C until use. For the crude homogenate preparation, the whole cerebral cortex was homogenized in 10 vol (w/v) of assay buffer (50 mM Tris HCl, pH 7.5 at 4°C) using an Ultra-Turrax, and was stored at –80°C for later use. For the membrane preparation, the whole cerebral cortex was homogenized in 10 vol (w/v) of ice-cold buffer (5 mM Tris HCl, 250 mM sucrose and 1mM EDTA, pH 7.5 at 4°C) using an Ultra-Turrax, and was then centrifuged at 1000 g for 10 min at 4°C. The supernatant was centrifuged at 50,000 g for 15 min at 4°C, and the resulting membrane pellet was resuspended in a cold assay buffer (50 mM Tris HCl, pH 7.5) and centrifuged again at 50,000 g for 15 min at 4°C. The final pellet was resuspended in the cold assay buffer and stored at –80°C for later use. Proteins were assayed according to the Bradford method (1976) (BioRad Laboratories, Inc.).
2.5.2. Radioligand binding assays

Crude tissue homogenates (400-500 μg protein) or tissue membrane preparations (200-300 μg protein) were incubated in duplicate for 45 min at 25°C with [3H]prazosin in 50 mM Tris HCl (pH 7.5) in a final volume of 1 ml. In competition experiments, crude homogenates or membranes were incubated with 0.2 nM [3H]prazosin, in the presence or absence of 15-20 concentrations of 5-methylurapidil (1 pM-100 μM) or BMY 7378 (10 pM-100 μM). In saturation experiments, membranes were incubated with 16 concentrations of [3H]prazosin (0.01-6 nM), in the presence or the absence of 100 μM GppNHp and in the presence of 1 mM MgCl2.

Experiments were terminated by rapid filtration through fiberglass filters (Schleicher and Schuell, GF 52) presoaked in 0.3% polyethyleneimine using a Brandel cell harvester (M24R). The filters were then washed three times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.5), and the filter-bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was measured in the presence of 10 μM phentolamine.

Competition radioligand binding experiments were sometimes conducted with the membranes obtained from rat cortical slices incubated for 30 min at 37°C in physiological solution (composition in mM: NaCl 118, KCl 4.75, CaCl2 1.8, MgCl2 1.2, KH2PO4 1.2, NaHCO3 25 and glucose 11, aerated with 95% O2/5% CO2) without or with noradrenaline (1 μM).

The data were analyzed by nonlinear regression using Prism version 4.0 (GraphPad Software; San Diego, California, U.S.A) to determine the dissociation constant (Kd), the maximum number of binding sites (Bmax) for saturation data, and
the Ki value for competition data, which was calculated from the IC<sub>50</sub> estimates according to the equation by Cheng and Prusoff (1973). Saturation data were fitted to hyperbolic functions (one or two sites) and competition data were fitted to mono or biphasic sigmoidal functions. The best fit to one-site or two-sites was evaluated using the F-test ($P < 0.05$).

2.6. Statistical analysis

Results are expressed as means ± S.E.M. for the n determinations obtained from different animals. Statistical significance was analyzed using the Student’s t test or one-way ANOVA and the Student-Newman-Keuls test (GraphPad Software; San Diego, California, U.S.A). A $P$-value of less than 0.05 was considered significant.

2.7. Drugs

The following drugs were obtained from Sigma (St. Louis, MO, USA): (-)-noradrenaline bitartrate, prazosin hydrochloride, litium chloride, GppNHP (5’-guanylimidodiphosphate), BMY 7378 (8-[2-[4-(2-Methoxyphenyl)-1-piperazyni l]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride), 5-methylurapidil, phentolamine mesylate. [7-methoxy-<sup>3</sup>H]prazosin (72-78 Ci mmol<sup>-1</sup>) and myo-[<sup>3</sup>H]inositol with PT6-271 stabilizer (80-110 Ci mmol<sup>-1</sup>) were obtained from Amersham Biosciences (Buckinghamshire, UK). Other reagents were of an analytical grade. All compounds were dissolved in distilled water.
3. Results

3.1. Accumulation of $[^3\text{H}]$-inositol phosphates

Noradrenaline (0.1 μM-1 mM) induced a concentration-dependent accumulation of $[^3\text{H}]$inositol phosphates in cerebral cortex slices (pEC$_{50} = 4.96 \pm 0.23$, maximum effect = 247.1 ± 12.2% with respect to basal $[^3\text{H}]$ inositol phosphate formation, n=3) (Fig. 1A).

To identify the subtypes implicated in α₁-adrenoceptor-induced inositol phosphate turnover, we tested the effect of different antagonists on basal and noradrenaline (100 μM) -induced $[^3\text{H}]$inositol phosphate accumulation. The antagonists tested were: prazosin, described as possessing a high affinity (pKi ~ 9.5-10.4) for α₁A-, α₁B- and α₁D-adrenoceptors and a low affinity (pK ~ 8-8.8) for α₁L-adrenoceptors; 5-methylurapidil, an α₁A-adrenoceptor selective antagonist showing a selectivity rank order of α₁A (pKi ~ 8.5-9.2) > α₁D (pKi ~ 7-8) > α₁B (pKi ~ 6.5-7) and with a low affinity for α₁L-adrenoceptor (pA$_2$ ~ 8); and BMY 7378, an α₁D-adrenoceptor-selective antagonist with a rank order of selectivity of α₁D (pKi ~ 8-9) > α₁A = α₁B (pKi ~ 6) (Ford et al., 1997; Michel et al., 1995; Morishima et al., 2008; Ohmura and Muramatsu, 1995; Oshita et al., 1991; Saussy et al., 1996; Schwinn et al., 1995).

Basal inositol phosphate accumulation (12.9 ± 0.99 dpm%, n=4) was not inhibited by prazosin (10 μM), 5-methylurapidil (10 μM) or BMY 7378 (10 μM) (90.7 ± 8.9%, 105.8 ± 2.5%, 99.4 ± 7.5% with respect to control, respectively) (Fig. 1B). However, all the antagonists produced concentration-dependent inhibition of the $[^3\text{H}]$inositol phosphate accumulation induced by 100 μM of noradrenaline (Fig.1C). The inhibition curves for prazosin fitted better to a one-site model with a low pKb value (pKb = 8.21), which highlights a participation of α₁L-adrenoceptor in the functional
response (Table 2). BMY 7378 monophasically inhibited $[^3]$Hinositol phosphate accumulation with a low affinity estimate (Table 2), which ruled out a major participation of $\alpha_{1D}$-adrenoceptors in the production of inositol phosphates. However, 5-methylurapidil inhibited $[^3]$Hinositol phosphate accumulation in a biphasic manner with high and low pKb values (Table 2) that are in accordance with those reported by Ford et al. (1997) for the cloned $\alpha_{1A/L}$- and $\alpha_{1B}$-adrenoceptor, respectively. The proportion of high affinity sites corresponding to $\alpha_{1A/L}$-subtype was approximately 64% (Table 2).

3.2. Determination of mRNA expression by real-time quantitative RT-PCR

The normalized values for mRNA levels encoding the $\alpha_{1A}$-, $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptor subtypes in copies/µg of total RNA are displayed in Fig. 2. The results indicate that all three $\alpha_1$-adrenoceptor subtypes were expressed in the rat cerebral cortex at different levels. The rank order was $\alpha_{1D}$ (~48%) > $\alpha_{1B}$ (~38%) >> $\alpha_{1A}$-adrenoceptor (~14%).

3.3. Western Blot studies

Immunoblotting experiments revealed protein expression of $\alpha_{1A}$-, $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptor subtypes in both membrane and cytosolic preparations of rat cerebral cortex. The distribution pattern of this expression differed; whereas the $\alpha_{1A}$- and $\alpha_{1B}$-adrenoceptor proteins were detected mainly in the membrane preparations, the $\alpha_{1D}$-adrenoceptor protein was detected predominantly in the cytosolic fractions (Fig. 3).

3.4. Radioligand binding studies: competition experiments
Competition experiments performed with 5-methylurapidil and BMY 7378 showed that both compounds inhibited the specific binding of 0.2 nM $[^3]$H)prazosin in a concentration-dependent manner, but the profiles of the inhibition curves differed according to the cortex preparation used (tissue membranes or crude homogenates) (Fig. 4).

The selective $\alpha_{1D}$-adrenoceptor antagonist BMY 7378 biphasically competed for $[^3]$H)prazosin binding to crude homogenates of rat cerebral cortex (Fig. 4A), in which it showed high and low affinity values (Table 3). The proportion of high affinity sites corresponding to the $\alpha_{1D}$-adrenoceptor subtype represented approximately 33% of the total specific binding (Table 3). However, BMY 7378 displayed a different profile in membrane preparations, in which it monophasically displaced $[^3]$H)prazosin binding with a single low affinity value, excluding the presence of the $\alpha_{1D}$-adrenoceptor subtype (Fig. 4A, Table 3).

5-Methylurapidil biphasically inhibited $[^3]$H)prazosin binding to membranes (Fig. 4B), thus revealing the existence of high and low affinity binding sites. These pKi values (Table 3) are in accordance with data from radioligand binding studies for the cloned $\alpha_{1A}$- and $\alpha_{1B}$-adrenoceptors, respectively (Ford et al., 1997; Michel et al., 1995; Schiwnn et al., 1995). The high affinity site percentage was 29.8% of the total specific binding.

To determine whether the major role of the $\alpha_{1A/L}$-adrenoceptor revealed by the functional study was due to a relative increase in the fraction of this subtype in the membrane after agonist treatment, rat cerebral cortical slices were pre-treated with 1 μM noradrenaline for 30 min. Tissue membranes were subsequently obtained and the effect of 5-methylurapidil and BMY 7378 on 0.2 nM $[^3]$H)prazosin binding was evaluated. The
BMY 7378 inhibition curves and the pKi values obtained were similar in non-treated and noradrenaline-pretreated membranes (Table 3; Fig. 4A). 5-Methylurapidil biphasically inhibited [³H]prazosin binding in control and pretreated membranes, revealing high and low affinity sites with similar pKi values (Tabla 3; Fig. 4B). However, the results showed a significantly higher proportion of high affinity sites corresponding to the α₁A-adrenoceptor subtype in membranes obtained from noradrenaline-pretreated slices than in those obtained from non-treated slices (Table 3).

3.5. Radioligand binding studies: saturation experiments

Saturation experiments were performed with [³H]prazosin at concentrations ranging from 0.01 to 6 nM in the presence or absence of 100 µM GppNHp to characterize the α₁L-adrenoceptor population present in the rat cerebral cortex. Fig. 5 presents representative data from these saturation experiments. The specific binding of [³H]prazosin was concentration-dependent and saturable under both experimental conditions. The Scatchard plot was curvilinear in the absence of 100 µM GppNHp (Fig. 5B), and the Hill coefficient was significantly different from unity (P<0.001, Table 4). This suggested the presence of high and low affinity binding sites for prazosin, as confirmed by the non-linear regression analysis of the binding isotherms. The dissociation constants (pKd) and maximal binding capacities (Bmax) for high and low sites are shown in Table 4. However, the Scatchard plot analysis of the binding data obtained in the saturation experiments performed in the presence of 100 µM GppNHp (Fig. 5D) produced a straight line (Table 4), which suggests the overwhelming presence of a single class of binding sites for [³H]prazosin in presence of GppNHp. The non-linear regression analysis fitted the data to a one-site model in which
the pKd value was similar to the high pKd value determined in the experiments performed without 100 μM GppNHp (Table 4). Moreover, as Table 4 shows, the maximal number of sites with high affinity for prazosin (Rhigh) in the presence of GppNHp was equal to the total amount of sites (R_{high} + R_{low}) obtained when the experiments were performed in the absence of the GTP analogue.
4. Discussion

The functional participation of the $\alpha_1$-adrenoceptor subtypes in the rat cerebral cortex has been determined by analyzing the effect of the antagonists prazosin (which discriminates high and low $\alpha_1$-adrenoceptor), 5-methylurapidil ($\alpha_{1A}$ selective) and BMY 7378 ($\alpha_{1D}$ selective) (Ford et al., 1997; Michel et al., 1995; Saussy et al., 1996; Schwinn et al., 1995) on the second messenger coupled to $\alpha_1$-adrenoceptor stimulation (inositol phosphate formation). Three conclusions can be drawn based on these results: 1) the low affinity estimates of BMY 7378 rules out a major participation of $\alpha_{1D}$-adrenoceptor in the inositol phosphate accumulation induced by noradrenaline; 2) the biphasic curve obtained with 5-methylurapidil (with high and low affinity estimates in a ratio of 64:36) suggests a mixed population of $\alpha_{1A/L}$- and $\alpha_{1B}$-adrenoceptors; and 3) the low potency of prazosin (pKb=8.21) confirms the contribution of the $\alpha_{1L}$ phenotype form in the functional response to $\alpha_1$-adrenoceptor stimulation. These results provide evidence of the major involvement of the $\alpha_{A/L}$-subtype in the second messenger signal mediated by activation of G-proteins in the membrane, and exclude a significant role for the $\alpha_{1D}$-adrenoceptor in this signalling pathway. Moreover, a constitutive activity of $\alpha_1$-adrenoceptor coupled to the inositol phosphate pathway can be ruled out, since basal inositol phosphate accumulation was not modified by $\alpha_1$-adrenoceptor antagonists (prazosin, 5-methylurapidil, BMY 7378).

The inability of the $\alpha_{1D}$-adrenoceptor to trigger inositol phosphate generation contrasts with the high mRNA and protein expression of this subtype. In fact, the rat cerebral cortex expresses mRNA of the three $\alpha_1$-adrenoceptor subtypes, and this expression is most pronounced in the $\alpha_{1D}$-adrenoceptor (Day et al., 1997; present study).
The presence of the three subtypes as proteins was also revealed by Western Blot analysis. Therefore, the inability of the \( \alpha_{1D} \)-adrenoceptor to couple to the inositol phosphate membrane signalling pathway may be related to the peculiar intracellular localization of this subtype, which contrasts with the predominant \( \alpha_{1A} \) and \( \alpha_{1B} \)-membrane expression reported in recombinant systems (Chalothorn et al., 2002; Hirasawa et al., 1997; McCune et al., 2000). At this point, we decided to assess if this cellular distribution pattern also exists in native tissues. To do this, we performed immunoblotting analysis of the three \( \alpha_{1} \)-adrenoceptors in membranes and cytosolic fractions of rat cerebral cortex. The results obtained indicate the predominant cytosolic localization of \( \alpha_{1D} \)-subtype in a native tissue, which contrasts with the general localization of \( \alpha_{1A} \) and \( \alpha_{1B} \)-adrenoceptors in the membrane. Therefore, we can conclude that the \( \alpha_{1D} \)-intracellular localization previously reported in recombinant systems (Chalothorn et al., 2002; McCune et al., 2000) is not an artefact of an artificial system, but rather a natural phenomenon.

The detection of \( \alpha_{1} \)-adrenoceptor proteins by radioligand binding assays confirms the presence of the three \( \alpha_{1} \)-adrenoceptors in the cerebral cortex and the intracellular localization of the \( \alpha_{1D} \)-subtype. In competition experiments performed in tissue membrane preparations, the monophasic BMY 7378 displacement curves and the low pKi obtained for this antagonist excluded a major presence of \( \alpha_{1D} \)-adrenoceptor in the membranes. However, the results obtained in crude tissue homogenates revealed that BMY 7378 biphasically displaced \(^3\text{H}\)prazosin binding; the high pKi value obtained (9.06) was similar to values previously reported for the cloned \( \alpha_{1D} \)-adrenoceptor (Saussy et al., 1996). Thus, our results provide further confirmation of the presence of the \( \alpha_{1D} \)-adrenoceptor in intracellular cytosolic compartments, since we detected this subtype in
crude homogenates and not in membrane preparations. This could explain the poor coupling characteristics of the $\alpha_{1D}$-adrenoceptor to membrane signals such as inositol phosphate turnover observed in a native tissue in the present study and previously described in recombinant systems (Schwinn et al., 1995; Theroux et al., 1996). The presence of $\alpha_{1A}$- and $\alpha_{1B}$ in the rat cerebral cortex was detected by competitive radioligand binding assays performed with 5-methylurapidil in rat cerebral membranes. This compound displaced $[^3H]$prazosin biphasically in membrane preparations, with high and low pKi values that correlate with those obtained for the cloned $\alpha_{1A}$- and $\alpha_{1B}$-subtype, respectively (Michel et al., 1995; Saussy et al., 1996; Schwinn et al., 1995). These results are similar to previously reported data (Madrero et al., 1996; Michel et al., 1993).

Taken as a whole, these results demonstrate the mRNA and protein expression of the three $\alpha_1$-adrenoceptors in the rat cerebral cortex. In addition, they reveal a different level of expression and distribution pattern for each one, which seems to determine the nature of their coupling to second messenger pathways. As described above, only the $\alpha_{1A/L}$- and $\alpha_{1B}$-adrenoceptor coupled to the inositol phosphate pathway, whereas the $\alpha_{1D}$-subtype did not. A surprising result was the major participation of $\alpha_{1A/L}$-adrenoceptor in inositol phosphate accumulation, despite its low mRNA and protein expression in the cerebral cortex. This could be due, at least in part, to differences in the internalization properties of $\alpha_{1A}$- and $\alpha_{1B}$-subtypes in recombinant systems: whereas the $\alpha_{1B}$-adrenoceptor undergoes robust agonist-induced endocytosis (Stanasila et al., 2008), a modest (Morris et al., 2004) or nonexistent internalization (Stanasila et al., 2008) has been described for the $\alpha_{1A}$-adrenoceptor.
Based on this hypothesis, after agonist stimulation in a native tissue expressing both subtypes, an increased proportion of $\alpha_{1A}$-adrenoceptor should be detected in the membrane. Thus, we determined the relative proportions of $\alpha_{1A}$- and $\alpha_{1B}$-adrenoceptors by radioligand binding experiments performed in membranes from rat cortical slices treated with or without noradrenaline. The results obtained revealed an increase in 5-methylurapidil high affinity sites in the tissue membranes after pre-treatment with the agonist, which could have been responsible, at least in part, for its significant involvement in the inositol phosphate response to $\alpha_{1}$-adrenoceptor stimulation.

The functional results also suggest that $\alpha_{1L}$ phenotype is involved in inositol phosphate turnover. It is accepted that this subtype is a phenotype form of the $\alpha_{1A}$-adrenoceptor (Ford et al., 1997; Gray et al., 2008; Marti et al., 2005; Muramatsu et al., 2008). Though it has been detected principally in functional studies, there are several reports that have demonstrated its presence using radioligand binding experiments. High and low affinity sites for prazosin were detected in membranes from rat cerebral cortex (Oshita et al., 1991) and rabbit liver (Ohmura and Muramatsu, 1995), and more recently in $\alpha_{1B}$-adrenoceptor knockout mouse liver in which $\alpha_{1A}$-adrenoceptor was upregulated (Deighan et al., 2004). However, recent studies using radioligand binding experiments have demonstrated the coexistence of $\alpha_{1A}$- and $\alpha_{1L}$-adrenoceptors in rat cerebral cortex segments but not in tissue membrane, suggesting that the $\alpha_{1L}$-phenotype is converted into the classic $\alpha_{1A}$-adrenoceptor upon homogenization (Morishima et al., 2008).

Similar results have been reported in rabbit ear artery (Hiraizumi-Hiraoka et al., 2004) and prostate (Morishima et al., 2007; Su et al., 2008). This apparent discrepancy between the results of different studies could be due to the different experimental conditions used. In recently published studies (Hiraizumi-Hiraoka et al., 2004;
Morishima et al., 2007, 2008; Su et al., 2008), saturation radioligand binding experiments were performed in tissue membranes in Krebs solution containing NaCl. It was found that Na$^+$ ions selectively regulated the spontaneous association between receptors and G proteins in a way that a higher proportion of the uncoupled form of the receptor is available in the presence of Na$^+$ (de Ligt et al., 2000).

Based on the above evidence, and considering that the $\alpha_{1L}$-adrenoceptor is the most evident phenotype when prazosin acts as an inhibitor of a functional response induced by an agonist, we hypothesized that the $\alpha_{1L}$ isoform represents a G-protein-coupled receptor state of the $\alpha_{1A}$-adrenoceptor that is favored when the agonist is present. To verify this hypothesis, radioligand saturation binding experiments were performed in membrane preparations in the presence or absence of GppNHp (a non-hydrolizable analogous of the GTP that uncouples receptor/G-protein complexes). The results obtained revealed the existence of sites with high and low affinity for prazosin, of which the latter type (low affinity) was not detected when saturation experiments were performed in the presence of GppNHp, indicating that this site corresponds to a G-protein-coupled state of the receptor. The fact that we did not observe a functional response in the absence of the agonist (prazosin, 5-methylurapidil and BMY 7378 did not decrease the basal accumulation of inositol phosphates) rules out the possibility that this isoform of the $\alpha_{1A/L}$-adrenoceptor has a constitutive activity. Therefore, according to the cubic ternary complex model (see Kenakin, 2004, for review), the low prazosin affinity sites ($\alpha_{1L}$) observed in radioligand binding studies may correspond to a population of an inactive isoform of $\alpha_{1A}$-adrenoceptors that are coupled to a G-protein.

In conclusion, the present work highlights mRNA and protein expression of the three $\alpha_1$-adrenoceptor subtypes ($\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$) in the rat cerebral cortex. The $\alpha_{1L}$-
adrenoceptor was also detected in functional and radioligand binding experiments but was absent in the presence of GppNHp, suggesting that the $\alpha_{1L}$-adrenoceptor corresponds to a form of the $\alpha_{1A}$ receptor that is coupled to a G-protein. The different experimental approaches used to characterize the $\alpha_1$-adrenoceptor subtypes reveal that the agonist-induced trafficking and the subcellular localization of these receptors in native tissue varies, which may determine the differences observed in their participation in a membrane signalling such as inositol phosphate accumulation.

**Acknowledgements**

This study was supported by the Spanish Dirección General de Programas y Transferencia de Conocimiento del Ministerio de Ciencia e Innovación (Grant SAF2004-01541 and SAF2007-62120); and Instituto de Salud Carlos III, Fondo de Investigaciones Sanitarias (Grant FIS PI070509). Vanessa Segura, Nicla Flacco and Eduardo Oliver are financed by the Spanish Ministry of Education and Science (Fellowships BES-2005-108383, AP-2005-5076 and AP-2004-3536).
References


Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099-3108.


Legends for figures

Fig. 1. Concentration-dependent stimulation of $[^3]H$inositol phosphate accumulation in response to noradrenaline (NA) in rat cerebral cortical slices. Data expressed as a percentage of the basal $[^3]H$inositol phosphate accumulation are presented as mean ± S.E.M. of 3 experiments performed in triplicate (A). The effect of $\alpha_1$-adrenoceptor antagonists on basal and 100 $\mu$M NA-induced $[^3]H$inositol phosphate accumulation in rat cerebral cortical slices. Data expressed as a percentage of the basal $[^3]H$inositol phosphate accumulation are presented as mean ± S.E.M. of 4-7 experiments (performed in triplicate). Basal= 2856 ± 219 dpm (n= 23) (B). Inhibition of NA-induced $[^3]H$inositol phosphate accumulation by $\alpha_1$-adrenoceptor antagonists in rat cerebral cortical slices. Data expressed as a percentage of the response to NA (100 $\mu$M) are presented as mean ± S.E.M. of 4-7 experiments performed in triplicate (C).

Pz: Prazosin 10 $\mu$M; 5-MU: 5-methylurapidil 10 $\mu$M; BMY: BMY 7378 10 $\mu$M.

*** $P < 0.001$ versus NA-induced inositol phosphate accumulation.

Fig. 2. $\alpha_1$-adrenoceptor subtypes mRNA in the rat cerebral cortex. Each value shown is the mean ± S.E.M. of 6 samples performed in triplicate and obtained from different animals.

Fig. 3. Immunoblotting detection of the $\alpha_1$-adrenoceptor subtypes present in membrane and cytosolic fractions of rat cerebral cortex. Quantitative protein expression measured by densitometric analysis and expressed as the ratio to actin (bar diagrams representing
mean ± S.E.M. of 3-5 samples obtained from different animals) (A), and representative blots (B).

* $P < 0.05$ versus membrane.

**Fig. 4.** Displacement of 0.2 nM $[\text{H}]$prazosin-specific binding to rat cerebral cortex membranes and crude homogenates by increasing concentrations of BMY 7378 (A) or of 5-methylurapidil (B). In some experiments membranes were obtained after treatment of rat cortical slices with noradrenaline 1 µM (NA-pretreated membranes) at 37°C for 30 min. Nonspecific binding was determined in the presence of 10 µM phentolamine. Data were presented as mean ± S.E.M. of $n$ individual experiments performed in duplicate, (see Table 3).

**Fig. 5.** Radioligand saturation-binding experiments with $[\text{H}]$prazosin in rat cerebral cortex membranes in the absence (A) or presence of GppNHp (100 µM) (C). The Scatchard plot of these data is shown in Fig. 5B and 5D, respectively. The dashed lines in Fig. 5B represent binding to the two sites. Each value is the mean of a duplicate determination. The figure is representative of either 6 (without GppNHp) or 3 experiments (with GppNHp).
Tables.

Table 1 Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequences(^a)</th>
<th>Positions(^b)</th>
<th>Product(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_{1A})-adrenoceptor</td>
<td>S 5'-CGA ATC CAG TGT CTT CGC AG-3' \ AS 5'-ACC ATG TCT CTG TGC TGT CCC-3'</td>
<td>1059-1078</td>
<td>100</td>
</tr>
<tr>
<td>(\alpha_{1B})-adrenoceptor</td>
<td>S 5'-GCT CCT TCT ACA TCC CGC TCG-3' \ AS 5'-AGG GGA GCC AAC ATA AGA TGA -3'</td>
<td>1018-1038</td>
<td>300</td>
</tr>
<tr>
<td>(\alpha_{1D})-adrenoceptor</td>
<td>S 5'-GAA GGT GAT GGG TTA TGG TG -3' \ AS 5'-GAA GCC ATA GCT GAA GCC T-3'</td>
<td>2321-2340</td>
<td>151</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>S 5'-GCA CCA CCA ACT GCT TAG CC-3' \ AS 5'-CTG AGT GGC AGT GAT GCC AT-3'</td>
<td>1298-1317</td>
<td>100</td>
</tr>
</tbody>
</table>

Oligonucleotide primer sequences for \(\alpha_{1A}\)-, \(\alpha_{1D}\)-adrenoceptor and GAPDH genes were in accordance with Marti et al. (2005) and those for \(\alpha_{1B}\)-adrenoceptor gene were in accordance with Scofield et al. (1995).

\(^a\) orientation, S: sense, AS: anti-sense.

\(^b\) positions depend on the corresponding mRNA sequence in the RefSeq NCBI Database (Pruitt and Maglott, 2001).

\(^c\) product size in base pairs.
**Table 2** Affinity estimates for $\alpha_1$-adrenoceptor antagonist inhibition curves obtained against noradrenaline (100 $\mu$M)-induced [$^3$H]inositol phosphate accumulation in rat cerebral cortical slices

<table>
<thead>
<tr>
<th></th>
<th>$pK_b$ high</th>
<th>$pK_b$ low</th>
<th>% high</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prazosin</strong></td>
<td>8.21 ± 0.07</td>
<td>-</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td><strong>5-methylurapidil</strong></td>
<td>8.40 ± 0.19</td>
<td>6.58 ± 0.24</td>
<td>64.1 ± 3.5</td>
<td>5</td>
</tr>
<tr>
<td><strong>BMY 7378</strong></td>
<td>6.07± 0.12</td>
<td></td>
<td>100</td>
<td>4</td>
</tr>
</tbody>
</table>

Mean ± S.E.M., n= number of experiments performed in triplicate.

$pK_b$ estimated according to Leff & Dougall (1993).

$pK_b$\textsubscript{high} and $pK_b$\textsubscript{low}: negative log of the inhibition constants (-log M) at high and low affinity sites.

% high: percentage of the receptor population with high affinity.
Table 3 Comparison of affinities of $\alpha_1$-adrenoceptor antagonists estimated from displacement of 0.2nM $[^3]$Hprazosin in rat cerebral cortex membranes or in total homogenates.

<table>
<thead>
<tr>
<th></th>
<th>BMY 7378</th>
<th>5-Methylurapidil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pK_i^{\text{high}}$</td>
<td>$pK_i^{\text{low}}$</td>
</tr>
<tr>
<td>Crude homogenates</td>
<td>9.06±0.22</td>
<td>6.58±0.19</td>
</tr>
<tr>
<td>Membranes</td>
<td>—</td>
<td>6.96±0.05</td>
</tr>
<tr>
<td>NA-pretreated membranes</td>
<td>—</td>
<td>6.89±0.07</td>
</tr>
</tbody>
</table>

Mean ± S.E.M., n= number of experiments performed in duplicate.

$pK_i^{\text{high}}$ and $pK_i^{\text{low}}$: negative log of the inhibition constants (-log M) at high and low affinity sites.

$\%$ high: percentage of the receptor population with a high affinity.

$^aP < 0.001$ compared to membranes.

NA-pretreated membranes: membranes obtained from rat cerebral cortical slices treated with 1 μM noradrenaline at 37°C for 30 min.
Table 4 Saturation \([^3H]\)prazosin binding to \(\alpha_1\)-adrenoceptors of rat cerebral cortex membranes

<table>
<thead>
<tr>
<th></th>
<th>pKd(_\text{high})</th>
<th>pKd(_\text{low})</th>
<th>R(_\text{high})</th>
<th>R(_\text{low})</th>
<th>Hill coefficient</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>-GppNHp</td>
<td>10.35 ± 0.09</td>
<td>8.84 ± 0.15</td>
<td>70.2 ± 10.5</td>
<td>40.0 ± 2.5</td>
<td>0.44 ± 0.08</td>
<td>6</td>
</tr>
<tr>
<td>+ GppNHp</td>
<td>10.16 ± 0.06</td>
<td>—</td>
<td>118.0 ± 6.8</td>
<td>—</td>
<td>0.87 ± 0.02</td>
<td>3</td>
</tr>
</tbody>
</table>

Mean ± S.E.M., n= number of experiments performed in duplicate.

pKd\(_\text{high}\) and pKd\(_\text{low}\): negative log of the equilibrium dissociation constants (-log M) at prazosin high and low affinity sites.

R\(_\text{high}\) and R\(_\text{low}\): maximal numbers of prazosin high and low affinity sites (fmol/mg protein).
mRNA (copies/μg of total RNA)

α₁A  α₁B  α₁D
Figure 3

A

Inmunodetectable protein

<table>
<thead>
<tr>
<th></th>
<th>Membrane</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1A</td>
<td><img src="image" alt="Graph for α1A" /></td>
<td><img src="image" alt="Graph for Cytosol" /></td>
</tr>
<tr>
<td>α1B</td>
<td><img src="image" alt="Graph for α1B" /></td>
<td><img src="image" alt="Graph for Cytosol" /></td>
</tr>
<tr>
<td>α1D</td>
<td><img src="image" alt="Graph for α1D" /></td>
<td><img src="image" alt="Graph for Cytosol" /></td>
</tr>
</tbody>
</table>

B

70 kDa - ![Western Blot for 70 kDa](image)

Actin - ![Western Blot for Actin](image)
Figure 4

A

Log [BMY 7378] (M) vs. (%)[3H]prazosin specific binding for Homogenates, Membranes, and NA-pretreated membranes.

B

Log [5-methylurapidil] (M) vs. (%)[3H]prazosin specific binding for Homogenates, Membranes, and NA-pretreated membranes.
Dear Dr. F. P. Nijkam,

After receiving the reviewer’s comments and checklist for style revision regarding our manuscript entitled “Alpha1-adrenoceptors in the rat cerebral cortex: new insights into the characterization of <alpha>1L- and <alpha>1D-adrenoceptors”, Number: EJP-31709R1, we have revised and corrected the text accordingly. We hope the manuscript is now suitable for publication.

Yours sincerely,

Specific comments:

Reviewer 2:

I would like to express my gratitude for the helpful comments of the reviewer.

Minor points:

1) p. 13, l. 17 and p. 18, l. 5: “Schwinn et al.”

This error has been corrected in p. 13, l. 17 and p. 18, l. 5.

2) p. 13, l. 18: The meaning of “Basal inositol phosphate accumulation (12.9 +/- 0.99 dpm %)” is unclear. The legend to Fig. 1 (p. 31, l. 9) gives a basal accumulation of 2856 +/- 219 dpm.

The legend to Fig. 1 gives the absolute value of basal inositol accumulation in dpm. This data was included in the previous revised version of the manuscript according to the reviewer’s suggestion. We have also expressed basal inositol phosphate accumulation as dpm% (p. 13, l. 18) because, as we explained in the “Materials and methods” section (p. 7, l. 21-23), we normalized the inositol phosphate radioactivities.

In order to clarify the term dpm%, we have re-written the above-cited paragraph (p. 7, l. 22), which now reads as follows:

“The accumulation of [3H]inositol phosphates was calculated as the percentage (dpm%) of [3H]inositol-labeled lipids in each sample to account for interexperimental variations in labelling and sample sizes…”

3) p. 15, l. 1: The verb is missing.

In the revised manuscript we have added the verb “showed” (p. 15, l. 2).

4) Table 3, first line: ”Comparison of affinities of .. antagonists”

As recommended by the referee, we have corrected the first line of Table 3.

Checklist for style revision:

- Rename heading 2 into 'Materials and methods'.
- Do not use AR as abbreviation. Write in full throughout the manuscript.
- Use P (not p)
- Type tables according EJP-style: do not use vertical lines.

The indications made in the Checklist have been addressed following the “Guide for Authors.”