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Short communication

# Low-affinity conditions for agonists increase the binding of the antagonist [$^3$H]RX821002 to the $\alpha_{2B/C}$-adrenoceptor subtypes in human brain and rat kidney

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**Abstract**

The mixture of 5′-guanylylimidodiphosphate (Gpp(NH)p)/EDTA/NaCl has been used to delineate low-affinity conditions for agonists binding to G-protein-linked receptors. The effects of this mixture on [$^3$H]RX821002 (2-methoxyidazoxan) binding to $\alpha_2$-adrenoceptors were evaluated in different tissues. The density of $\alpha_2$-adrenoceptors in the presence of the mixture was 11, 78 and 60% higher in human cortex (predominant $\alpha_{2A}$), human caudate ($\alpha_{2A} + \alpha_{2C}$) and rat kidney ($\alpha_{2A} + \alpha_{2B}$), respectively, than in its absence. In rat kidney, masking of $\alpha_{2B}$-adrenoceptors by ARC239 (2-[2-[4-(*o*-methoxyphenyl)-piperazin-1-yl]-ethyl]-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione) (50 nM) or masking of $\alpha_{2A}$-adrenoceptors by BRL44408 (2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole) (100 nM) demonstrated that the increase was in the $\alpha_{2B}$-adrenoceptor but not in the $\alpha_{2A}$-adrenoceptor subtype. © 1997 Elsevier Science B.V.

*Keywords:* RX821002 (2-methoxyidazoxan); $\alpha_2$-Adrenoceptor subtype; Gpp(NH)p; $Na^+$

## 1. Introduction

$\alpha_2$-Adrenoceptors are a group of receptors linked to inhibitory $G_{i/o}$ proteins. These receptors show high- and low-affinity states for agonist drugs while antagonist drugs display similar affinity at both receptor conformations (Hoffman and Lefkowitz, 1980). Guanine nucleotides and $Na^+$ regulate agonist binding to $\alpha_2$-adrenoceptors and promote the low-affinity state (Michel et al., 1980). Since the presence of two different affinity conformations of $\alpha_2$-adrenoceptors makes the interpretation of agonist binding data difficult, a mixture of Gpp(NH)p/EDTA/NaCl is usually included in the incubation buffer as the optimal condition to fully eliminate the high-affinity state for agonists of $\alpha_2$-adrenoceptors (Uhlén and Wikberg, 1991). However, some studies have reported that $Na^+$ and guanine nucleotides also modulate antagonist binding to $\alpha_2$-adrenoceptors (Limbird et al., 1982; Jagadeesh et al., 1990; MacKinnon et al., 1993).

Currently the $\alpha_2$-adrenoceptors are subdivided into three subtypes, $\alpha_{2A}$-, $\alpha_{2B}$- and $\alpha_{2C}$-adrenoceptors, which are expressed in different proportions in a wide range of tissues (Bylund, 1992). In the human brain, the $\alpha_{2A}$-adrenoceptor subtype is predominant in the frontal cortex whereas the $\alpha_{2C}$-adrenoceptor is present in the caudate (Grijalba et al., 1996). The rat kidney possesses both $\alpha_{2A}$-and $\alpha_{2B}$-adrenoceptors with a major proportion of them being of the $\alpha_{2B}$-adrenoceptor subtype (Callado et al., 1996).

The aim of the present study was to evaluate the effects of low-affinity conditions on the binding of [$^3$H ]RX821002 to the $\alpha_2$-adrenoceptors expressed in different tissues and to assess whether such alterations are selective for any of the described $\alpha_2$-adrenoceptor subtypes.

## 2. Materials and methods

Human brains were obtained at autopsy, dissected and stored at −70°C. Male Sprague–Dawley rats (230–260 g) were killed and their kidneys were excised and stored at −70°C until assay.

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After thawing, $P_2$ fractions of membranes from human prefrontal cortex and caudate head, and from rat kidney were prepared as described (Callado et al., 1996; Grijalba et al., 1996). The final pellet was resuspended in an appropriate volume of Tris incubation buffer (50 mM Tris-HCl; pH 7.5) and protein levels were measured using bovine serum albumin as the standard.

The binding assays for [$^3$H]RX821002 were performed as described previously (Callado et al., 1996; Grijalba et al., 1996) by incubating 0.7–1.3 mg protein/ml of the membranes in 550 μl of a solution containing Tris incubation buffer with [$^3$H]RX821002 and different drugs for 30 min at 25°C. All the experiments were performed in the presence or absence of a mixture containing Gpp(NH)p (100 μM), EDTA (1 mM) and NaCl (140 mM). Incubations were stopped by diluting the samples and then filtering and washing them on glass fiber filters which had been presoaked with 0.5% polyethylenimine. The filters were counted for radioactivity by liquid scintillation spectrometry. Non-specific binding, as estimated in the presence of $10^{-5}$ M (−)-adrenaline, ranged from 5 to 39%.

Analyses of saturation isotherms ($K_d$, dissociation constant; $B_{max}$, maximum density of binding sites) were performed by computer-assisted non-linear regression, using the EBDA-LIGAND programs. Saturation data are expressed as the best fit value ± standard error (S.E.) obtained from a computer-assisted simultaneous coanalysis and the differences between binding conditions were made statistically after constraining the two sets of data to share $B_{max}$ or $K_d$ parameters ($F$-test). In masking experiments, values are given as the mean ± standard error of the mean (S.E.M.) and statistical differences between groups were determined by paired two-tailed $t$-tests. The level of significance was at $P = 0.05$.

[$^3$H]RX821002 (1,4-[6,7(n)-[$^3$H]benzodioxan-2-methoxy-2-yl)-2-imidazoline HCl; 53–62 Ci/mmol) was purchased from Amersham (Amersham, UK). (−)-Adrenaline bitartrate and Gpp(NH)p (5′-guanylylimidodiphosphate) were from Sigma (St. Louis, MO, USA); 2-[2-[4-(*o*-methoxyphenyl)-piperazin-1-yl]-ethyl]-4,4-dimethyl-1,3 (2H,4H)-isoquinolindione HCl (ARC239) was from K. Thomae (Biberach, Germany); 2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole HCl (BRL44408) was from Beecham (Harlow, UK).

## 3. Results

The specific binding of [$^3$H]RX821002 (0.125–16 nM, eight concentrations) to membranes from human prefrontal cortex, human caudate and rat kidney was saturable with $K_d$ values in the nanomolar range (Table 1). Non-linear analyses of saturation data showed a best fit in all the cases to a single binding site model (Table 1).

The addition to the assay of a mixture containing the stable GTP-analogue Gpp(NH)p (100 μM), EDTA (1 mM) and NaCl (140 mM) resulted in a increase of $B_{max}$ values that was statistically significant in the three regions studied (Table 1). The increases were greater in the human caudate (78%; $P < 0.001$) and the rat kidney (60%; $P < 0.001$) than in the human cortex (11%; $P < 0.005$). The affinity of [$^3$H]RX821002 binding was not significantly affected by the mixture in any of the three regions (Table 1).

In order to determine whether the increased number of specific [$^3$H]RX821002 binding sites was in relation with any concrete subtype of $\alpha_2$-adrenoceptors, masking experiments with subtype-selective drugs were performed with rat kidney membranes, using a single concentration of [$^3$H]RX821002 (2 nM) near to $K_d$ values (Table 1).

Under basal conditions (without any masking drug in the assay), Gpp(NH)p/EDTA/NaCl increased [$^3$H]RX821002 binding to the rat kidney membranes as compared with the binding obtained in the absence of the mixture (+51 ± 5%; $P < 0.001$) (Fig. 1). As previously described (Callado et al., 1996), the $\alpha_{2A}$-adrenoceptor-selective antagonist BRL44408 (100 nM) was added to the assay in order to mask the $\alpha_{2A}$-adrenoceptor population in rat kidney. Using this approach, the addition of the mixture induced a higher [$^3$H]RX821002 specific binding than that obtained in the absence of the mixture (+53 ± 9%; $P < 0.01$) (Fig. 1). Conversely, in the presence of the $\alpha_{2B/C}$-adrenoceptor-selective antagonist ARC239 (50 nM), to selectively mask $\alpha_{2B}$-adrenoceptors, the binding of [$^3$H]RX821002 to $\alpha_{2A}$-adrenoceptors was not significantly

Table 1
Effects of Gpp(NH)p/EDTA/NaCl-containing mixture on specific [$^3$H]RX821002 binding to $\alpha_2$-adrenoceptors in three different regions

| | Human cortex | | Human caudate | | Rat kidney | |
|---|---|---|---|---|---|---|
| | $B_{max}$ (fmol/mg protein) | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) | $K_d$ (nM) |
| Control | 176 ± 5 | 0.89 ± 0.04 | 50 ± 4 | 1.44 ± 0.16 | 57 ± 5 | 1.51 ± 0.16 |
| +Mixture | 197 ± 6 [a] | 0.83 ± 0.03 | 89 ± 4 [b] | 1.19 ± 0.08 | 91 ± 15 [b] | 1.30 ± 0.31 |

Membranes of each tissue were incubated with [$^3$H]RX821002 (0.125–16 nM, eight concentrations) for 30 min at 25°C. The mixture containing Gpp(NH)p (100 μM), EDTA (1 mM) and NaCl (140 mM) was added directly to the assay. Non-specific binding was estimated as [$^3$H]RX821002 binding in the presence of $10^{-5}$ M (−)-adrenaline. Data are the estimated best fit ± S.E. of 3–5 independent experiments and were determined by the EBDA-LIGAND programs. These S.E. values obtained from non-linear regression were not used in further formal statistical calculations.
[a] $P < 0.005$ and [b] $P < 0.001$ compared to corresponding control values ($F$-test).

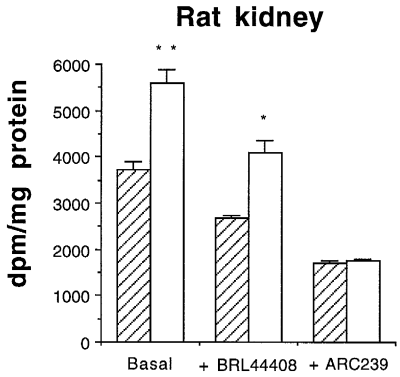


Fig. 1. Specific binding of [$^3$H]RX821002 (2 nM) to rat kidney membranes in absence (hatched columns) or presence (white columns) of a mixture containing Gpp(NH)p (100 μM), EDTA (1 mM) and NaCl (140 mM). Basal columns represent experiments performed without any masking drug in the assay, +BRL44408 represents inclusion in the assay of 100 nM BRL44408 in order to mask $\alpha_{2A}$-adrenoceptors, and +ARC239 indicate inclusion of 50 nM ARC239 to mask $\alpha_{2B}$-adrenoceptors. Values are means ± S.E.M. of 4–10 independent experiments and are expressed in dpm/mg protein. * $P < 0.01$ and * * $P < 0.001$ versus values of [$^3$H]RX821002 specific binding in the absence of the mixture (Student's *t*-test).

different in the presence or absence of the mixture (+3 ± 0.2%) (Fig. 1).

The non-specific binding was not significantly affected in any of the assays by the addition of the mixture Gpp(NH)p/EDTA/NaCl, indicating that only the specific binding of [$^3$H]RX821002 to $\alpha_2$-adrenoceptors was modified by the mixture.

## 4. Discussion

The present study initially showed that the addition of Gpp(NH)p/EDTA/NaCl significantly increases the specific binding of the antagonist [$^3$H]RX821002 to $\alpha_2$-adrenoceptors.

The non-hydrolyzable GTP-analogue, Gpp(NH)p, and the chelator of divalent cations, EDTA, are used to bring $\alpha_2$-adrenoceptors into the low-affinity state for agonists. However, in some cases competition curves for full agonists such as (−)-adrenaline are still shallow and could be resolved into two-site fits, indicating that some agonist/receptor/G-protein complexes are still being formed (Uhlén and Wikberg, 1991). NaCl concentrations higher than 30 mM cause a rightward-shift of the agonist competition isotherms (Schloos et al., 1987) in an additive manner to the effect of the Gpp(NH)p (Schloos et al., 1987; Jagadeesh et al., 1990). Thus, a Gpp(NH)p/EDTA/NaCl-containing buffer is necessary to completely eliminate the high-affinity state for agonists of the $\alpha_2$-adrenoceptors (Asakura et al., 1985; Jagadeesh et al., 1990; Uhlén and Wikberg, 1991).

Previous studies have shown that the addition of Gpp(NH)p to the binding assay significantly increases the amount of specific binding of antagonist radioligands to dopamine receptors (Hall et al., 1992), adenosine receptors (Yeung and Green, 1983) and $\alpha_2$-adrenoceptors (Jagadeesh et al., 1990). In the same manner, $Na^+$ has been described to increase the apparent density of $\alpha_2$-adrenoceptors, as evaluated with the antagonist [$^3$H]yohimbine (Limbird et al., 1982), although no changes have been reported for the diastereoisomer [$^3$H]rauwolscine (Jagadeesh et al., 1990).

In our study the increase in $\alpha_2$-adrenoceptor density produced by Gpp(NH)p/EDTA/NaCl and evaluated with the antagonist [$^3$H]RX821002 was different for each specific tissue. In this way, the increase observed in the human cortex, where the $\alpha_{2A}$-adrenoceptor subtype is predominant (Grijalba et al., 1996), was lower than that in the human caudate or in the rat kidney, where $\alpha_{2C}$- and $\alpha_{2B}$-adrenoceptors are the predominant subtypes, respectively (Callado et al., 1996; Grijalba et al., 1996). The data obtained from masking experiments in a tissue, rat kidney, that expresses two different $\alpha_2$-adrenoceptor subtypes appear to confirm the findings. After $\alpha_{2A}$-adrenoceptors in rat kidney were masked with the selective antagonist BRL44408, [$^3$H]RX821002 binding to the remaining $\alpha_{2B}$-adrenoceptors was significantly increased in the presence of the mixture. In contrast, [$^3$H]RX821002 binding to $\alpha_{2A}$-adrenoceptors, evaluated by masking of $\alpha_{2B}$-adrenoceptors with the selective antagonist ARC239, was not different in the presence or absence of the Gpp(NH)p/EDTA/NaCl-containing mixture. So, the low-affinity conditions obtained with the mixture seem to specifically increase [$^3$H]RX821002 binding to $\alpha_{2B}$-adrenoceptors without altering binding to the $\alpha_{2A}$-adrenoceptor subtype.

Our results are consistent with those of experiments performed in tissues expressing different $\alpha_2$-adrenoceptor subtypes where 100 mM NaCl selectively increased the density of sites labelled with the antagonist [$^3$H]RS-15385-197 in neonatal rat lung (exclusive presence of $\alpha_{2B}$-adrenoceptors) but not in human platelets (exclusive presence of $\alpha_{2A}$-adrenoceptors), without changing affinity values (MacKinnon et al., 1993). In the same sense, a buffer containing GTP and/or NaCl did not alter [$^3$H]RX821002 binding density in rat cortex, where $\alpha_{2A}$-adrenoceptors are predominant (Erdbrügger et al., 1995), nor in human fat cell $\alpha_{2A}$-adrenoceptors (Galitzky et al., 1990; Langin et al., 1990).

The specific effect of the Gpp(NH)p/EDTA/NaCl-containing mixture on $\alpha_{2B}$-adrenoceptors, and probably on $\alpha_{2C}$-adrenoceptors, may be related to the reported results indicating that at least 40% of $\alpha_{2B}$-adrenoceptors exist as a precoupled receptor/G-protein complex which fails to bind [$^3$H]antagonists (Shi and Deth, 1994). It has been proposed that $Na^+$ and Gpp(NH)p reduce the proportion of precoupled receptors, so when binding is carried out in the presence of a combination of $Na^+$ and Gpp(NH)p, which promotes dissociation of the R/G complex, evident increases in the density without changes in affinity are

observed (Shi and Deth, 1994). In this context, $\alpha_2$-adrenoceptor subtypes can be expected to differ in their extent of precoupling and this fact could explain the differential effect caused by the mixture on the [$^3$H]RX821002 binding density for each different $\alpha_2$-adrenoceptor subtype assessed. However, some authors have speculated that RX821002, and other $\alpha_2$-adrenoceptor antagonists, could have 'reverse antagonistic' properties (Erdbrügger et al., 1995).

In conclusion, the low-affinity conditions obtained by addition of Gpp(NH)p, EDTA and NaCl to the binding assay appear to selectively increase [$^3$H]RX821002 specific binding to the $\alpha_{2B/C}$-adrenoceptor subtypes in human brain and rat kidney.

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## References

Asakura, M., Tsukamoto, T., Imafuku, J., Matsui, H., Ino, M., Hasegawa, K., 1985. Quantitative analysis of rat brain $\alpha_2$-receptors discriminated by [$^3$H]clonidine and [$^3$H]rauwolscine. Eur. J. Pharmacol. 106, 141–147.

Bylund, D.B., 1992. Subtypes of $\alpha_1$- and $\alpha_2$-adrenergic receptors. FASEB J. 6, 832–839.

Callado, L.F., Gabilondo, A.M., Meana, J.J., 1996. [$^3$H]RX821002 (2-methoxyidazoxan) binds to $\alpha_2$-adrenoceptor subtypes and a non-adrenoceptor imidazoline binding site in rat kidney. Eur. J. Pharmacol. 316, 359–368.

Erdbrügger, W., Raulf, M., Otto, T., Michel, M.C., 1995. Does [$^3$H]2-methoxy-idazoxan (RX821002) detect more alpha-2-adrenoceptor agonist high-affinity sites than [$^3$H]rauwolscine? A comparison of nine tissues and cell lines. J. Pharmacol. Exp. Ther. 273, 1287–1294.

Galitzky, J., Larrouy, D., Berlan, M., Lafontan, M., 1990. New tools for human fat cell alpha-2A adrenoceptor characterization. Identification on membranes and on intact cells using the new antagonist [$^3$H]RX821002. J. Pharmacol. Exp. Ther. 252, 312–319.

Grijalba, B., Callado, L.F., Meana, J.J., García-Sevilla, J.A., Pazos, A., 1996. $\alpha_2$-Adrenoceptor subtypes in the human brain: A pharmacological delineation of [$^3$H]RX-821002 binding to membranes and tissue sections. Eur. J. Pharmacol. 310, 83–93.

Hall, H., Halldin, C., Sedvall, G., 1992. Gpp(NH)p stimulates [$^3$H]raclopride binding to homogenates from human putamen and accumbens. Neurosci. Lett. 136, 79–82.

Hoffman, B.B., Lefkowitz, R.J., 1980. Radioligand binding studies of adrenergic receptors: New insights into molecular and physiological regulation. Annu. Rev. Pharmacol. Toxicol. 20, 581–608.

Jagadeesh, G., Cragoe, E.J., Deth, R.C., 1990. Modulation of bovine aortic alpha-2 receptors by $Na^+$, 5′-guanylylimidodiphostate, amiloride and ethylisopropylamiloride: Evidence for receptor G-protein precoupling. J. Pharmacol. Exp. Ther. 252, 1184–1196.

Langin, D., Paris, H., Lafontan, M., 1990. Binding of [$^3$H]idazoxan and of its methoxy derivative [$^3$H]RX821002 in human fat cells: [$^3$H]idazoxan but not [$^3$H]RX821002 labels additional non-$\alpha_2$-adrenergic binding sites. Mol. Pharmacol. 37, 876–885.

Limbird, L.E., Speck, J.L., Smith, S.K., 1982. Sodium ion modulates agonist and antagonist interactions with the human platelet alpha$_2$-adrenergic receptor in membrane and solubilized preparations. Mol. Pharmacol. 21, 609–617.

MacKinnon, A.C., Spedding, M., Brown, C.M., 1993. Sodium modulation of $^3$H-agonist and $^3$H-antagonist binding to $\alpha_2$-adrenoceptor subtypes. Br. J. Pharmacol. 109, 371–378.

Michel, T., Hoffman, B.B., Lefkowitz, R.J., 1980. Differential regulation of the $\alpha_2$-adrenergic receptor by $Na^+$ and guanine nucleotides. Nature 288, 709–711.

Schloos, J., Wellstein, A., Palm, D., 1987. Agonist binding at alpha$_2$-adrenoceptors of human platelets using $^3$H-UK-14,304: Regulation by Gpp(NH)p and cations. Naunyn-Schmiedebergs Arch. Pharmacol. 336, 48–59.

Shi, A., Deth, R.C., 1994. Precoupling of alpha-2B adrenergic receptors and G-proteins in transfected PC-12 cell membranes: Influence of pertussis toxin and lysine-directed cross-linker. J. Pharmacol. Exp. Ther. 271, 1520–1527.

Uhlén, S., Wikberg, J.E.S., 1991. Rat spinal cord $\alpha_2$-adrenoceptors are of the $\alpha_{2A}$-subtype: Comparison with $\alpha_{2A}$- and $\alpha_{2B}$-adrenoceptors in rat spleen, cerebral cortex and kidney using $^3$H-RX821002 ligand binding. Pharmacol. Toxicol. 69, 341–350.

Yeung, S.M.H., Green, R.D., 1983. Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5′-guanylylimidodiphosphate or *N*-ethylmaleimide. J. Biol. Chem. 258, 2334–2339.