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Functional autoradiography and gene expression analysis applied to the characterization of the α_2-adrenergic system in the chicken brain

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ABSTRACT

Here we report a functional autoradiographic study of [^{35}S]GTPγS binding induced by α_2-adrenoceptor activation in chicken brain tissue sections using both 10^{-4} M UK 14304 (bromoxidine or brimonidine) and 10^{-6} M epinephrine as α_2-adrenoceptor agonists. Assays were performed using two different incubation buffers: glycylglycine or Tris–HCl. Changes in the [^{35}S]GTPγS basal binding values were detected, and different [^{35}S]GTPγS specific binding values were also obtained depending on the buffer used for each drug. The best results were obtained with epinephrine in Tris–HCl, with slightly higher stimulation values than the observed with UK 14304 in glycylglycine buffer. The effect of the addition of adenosine deaminase to the incubation buffer was also tested. This effect decreasing basal binding in chicken was very small when compared to mammals, according with differences found in adenosine 1 receptor expression levels. Structures presenting α_2-adrenoceptor-mediated $G_{i/o}$ protein stimulation fitted with areas previously described as enriched in α_2-adrenoceptors in chicken brain, and their homologous areas in mammals. These data confirm the specificity of the results and reinforce the implication of the α_2-adrenoceptors in the function of these brain nuclei. On the other hand, the expression level of the different α_2-adrenoceptor subtypes was tested with real-time PCR. Contrasting with the α_2-adrenoceptor subtype distribution previously described with radioligand competition assays, where α_{2A} was the predominant α_2-adrenoceptor subtype ($\geq$75%); in the present work, the ratio of α_{2A}:$\alpha_{2B/C}$ gene expression was lower than expected both in telencephalon, tectum opticum, and cerebellum.

1. Introduction

Avian biological diversity and its complex social behaviour make an excellent model for studying neuronal functions such as seasonal neurogenesis, apoptosis, learning, neuronal proliferation and plasticity (Bottjer and Arnold, 1997; Tramontin and Brenowitz, 2000). In previous studies we determined the pharmacological profile and distribution of the α_2-adrenoceptor subtypes in the chicken central nervous system (CNS), showing that they are highly conserved receptors (Fernandez-Lopez et al., 1990, 1997) and confirming the presence of the three different α_2-adrenoceptor subtypes described for the human brain: α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptors (Diez-Alarcia et al., 2006). Those data also showed a similar pharmacological profile between chicken and rat α_2-adrenoceptors in the brain, and a highly conserved anatomical distribution of these receptors in birds and mammals CNS. Although chicken genes codifying for α_2-adrenoceptors have not yet been sequenced, the comparison of primary and secondary structures of α_2-adrenoceptors cloned for different species of mammals and fish shows a high correlation coefficient (Svensson et al., 1993; Ruuskanen et al., 2005). Now, in an attempt to better characterize the α_2-adrenoceptor system in this species, we setup for the first time the [^{35}S]GTPγS autoradiography for α_2-adrenoceptors in chicken brain tissue sections. Moreover, we also report the expression levels of the three α_2-adrenoceptor subtypes determined by real-time RT-PCR.

As members of the G protein-coupled receptors (GPCRs) family, the α_2-adrenoceptors interaction with heterotrimeric G proteins

Abbreviations: GPCR, G protein-coupled receptor; CNS, central nervous system; [^{35}S]GTPγS, [^{35}S]guanylyl-5′-O-(γ-thio)-triphosphate; ADA, adenosine deaminase; E, epinephrine; ASB, agonist stimulated binding; NSB, non-specific binding; A1R, adenosine A1 receptor.

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constitutes an essential part of their functional activity. When an agonist drug binds to a GPCR, and this complex interacts with a G protein, the exchange of GDP by GTP in the alpha subunit is induced, and the dissociation of the G protein complex in Gα and Gβγ dimer is triggered. Both units are able to initiate different intracellular signalling pathways by activating or inhibiting enzymes such as adenylyl cyclase, phospholipases, ionic channels, etc. The activation of GPCRs can be assessed resorting to different experimental approaches; one of them is the stimulation of the [^{35}S]GTPγS binding assay. This technique is based on the decrease of the Gα subunit affinity for the GDP induced by the interaction of the G protein with an activated receptor, resulting in an increase in the apparent affinity of the alpha subunit for GTP, and its analogues. In this way, the use of [^{35}S]GTPγS, a non-hydrolyzable GTP analogue, allows to measure the efficacy of an agonist in inducing the receptor activation and its coupling to G proteins (Happe et al., 2000).

The quantification of G protein activation induced by the interaction of a given agonist with its receptor was first set-up on tissue membrane homogenates by using [^{35}S]GTPγS binding studies (Hilf et al., 1989; Traynor and Nahorski, 1995; Gonzalez-Maeso et al., 2000). Then, this technique was optimised to identify activated G proteins in brain tissue slices (Sim et al., 1995), allowing a detailed analysis of the anatomic distribution of the [^{35}S]GTPγS binding induced by agonist. However, it must be taken into account that the stimulation of [^{35}S]GTPγS binding is mainly due to $G_{i/o}$ proteins (Carty and Iyengar, 1994; Sim et al., 1997; Waeber and Moskowitz, 1997), which are the most abundant subtype within the CNS (Sternweis and Robishaw, 1984), and different dissociation rates exist between GDP and the distinct Gα subunit subtypes ($G\alpha_i > G\alpha_s$) (Carty and Iyengar, 1994). A noticeable feature of this approach is the heterogeneously distributed signal obtained under basal assay conditions in many CNS regions. Endogenous adenosine, acting via A1 receptors, is the most important identified factor contributing to these [^{35}S]GTPγS basal binding values in the rat brain (Laitinen, 1999; Moore et al., 2000). However, despite elimination of the adenosine signal, several "hot spots" loci (e.g. the hypothalamus) present high local basal G protein activity, and endogenous ligands responsible for this activity are yet to be identified (Laitinen, 1999). One of the aims of this study was to check the contribution of the A1 receptors activation to the basal and stimulated [^{35}S]GTPγS binding in chicken by carrying out functional autoradiographic assays in the presence and in the absence of adenosine deaminase (ADA).

The [^{35}S]GTPγS autoradiographic functional assays have been successfully performed on different receptor systems (Sim et al., 1997; Waeber and Moskowitz, 1997; Newman-Tancredi et al., 2000; Rodriguez-Puertas et al., 2000; Happe et al., 2000; Pilar-Cuellar et al., 2005; Alonso-Ferrero et al., 2006) and the anatomical distribution obtained has always been similar to that observed using quantitative autoradiographic assays (Happe et al., 2001). However, technical difficulties have been found for some receptors systems, mainly in tissues, when taking this pharmacological approach (Sim et al., 1997; Waeber and Moskowitz, 1997). Thus [^{35}S]GTPγS binding induced by α_2-adrenoceptor agonists seems to be easier to detect in cell line studies where α_2-adrenoceptor density is very high (Wise et al., 1997; Pauwels and Tardif, 2002). In contrast, those performed on native tissues, where expression levels are very low, present relatively modest [^{35}S]GTPγS specific binding even in the receptor-enriched regions (Happe et al., 2000, 2001). Considering that agonist-induced [^{35}S]GTPγS binding depends on many factors, such as receptor density, catalytic efficacy of the receptor-G protein complex (Sim et al., 1997), [^{35}S]GTPγS basal binding values (Happe et al., 2001); the tuning and characterization of the specific assay conditions for each different receptor system, and each sample conditions, species, is very important to achieve the best possible results. A further aim of this study is to compare different conditions of incubation to improve the [^{35}S]GTPγS specific binding mediated by α_2-adrenoceptor activation in chicken brain. Thus, in this study we compare different conditions for functional autoradiography assays on the α_2-adrenoceptor agonist stimulated [^{35}S]GTPγS binding in avian brain tissue sections, and we also describe the anatomical distribution of this stimulation using two different agonists: epinephrine (E) and UK 14304 (brimonidine and also bromoxidine). All the assays were carried out simultaneously in rat brain tissue sections to control the smooth running of the experimental conditions.

Finally, since a major problem in pharmacologically discriminating α_2-adrenoceptor subtypes is the lack of subtype-selective ligands (Sallinen et al., 2007; Gentili et al., 2007), the autoradiographic characterization of the anatomical distribution of the different α_2-adrenoceptor subtypes is very complicated. Instead of this pharmacological approach, we use herein real-time RT-PCR assays in an attempt to describe the distribution of mRNA of the α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptor subtypes in several structures of the chicken brain.

2. Experimental procedures

All the animals used in this work were treated in accordance with the European Communities Council Directive of 1986 November 24th (86/609/EEC).

For autoradiographic studies, five male one-month-old chickens (*Gallus gallus*) and five male Sprague–Dawley rats were used. Animals were killed by decapitation; brains were rapidly removed, frozen in liquid nitrogen, and stored at −80 °C until use. Coronal (chicken) or sagittal (rat) sections, 12 μm thick, obtained with a cryostat were mounted onto gelatin-coated slides and stored at −80 °C. Sections were used with less than two weeks since cut. One adjacent section of each series was collected and stained with cresyl violet to identify brain regions by comparison with chicken (Kuenzel and Masson, 1998) and rat (Paxinos and Watson, 1998) brain atlas. Unless specified, the nomenclature used for the chicken brain nuclei is from (Reiner et al., 2004).

For real-time PCR assays, four male one-month-old chickens and five male Sprague–Dawley rats were used. Animals were killed by decapitation and brains were rapidly removed and dissected. Chicken telencephalon, tectum opticum, wulst (structure including hyperpallium accesorium + intercallatum + densocellulare), and cerebellum were isolated while for rat gene expression assays, only cortex and cerebellum were dissected. All the samples were obtained under dissecting microscope, rapidly frozen in liquid nitrogen and maintained at −80 °C until further processing.

2.1. Functional autoradiography

Two different α_2-adrenoceptor agonists were used to induce the activation of α_2-adrenoceptors in chicken brain tissue sections. We used as a model the protocol described by Sim et al. (1995), and then tried different buffers and incubation conditions described later to improve α_2-adrenoceptor mediated [^{35}S]GTPγS autoradiographic results in mammalian CNS (Happe et al., 2001).

Sections were thawed for 5 min at room temperature (RT) and dipped in pre-incubation buffer (Tris–HCl or glycylglycine 50 mM, $MgCl_2$ 3 mM, EGTA 1 mM, NaCl 100 mM and GDP 2 mM, pH 7.5) for 30 min RT to remove endogenous ligands. Then, sections were incubated in the incubation buffer (Tris–HCl or glycylglycine 50 mM, $MgCl_2$ 3 mM, EGTA 1 mM, NaCl 100 mM, GDP 2 mM and DTT 0.2 mM, pH 7.5) containing the radioligand ([^{35}S]GTPγS 0.1 nM, Perkin Elmer, UK) for 4 h at RT, and finally dipped and washed for 5 min in washing buffer (Tris–HCl or glycylglycine 50 mM, pH 7.5) at 4 °C. Lastly, sections were dipped in distilled water at 4 °C and dried overnight in a cold air stream. Receptor-stimulated [^{35}S]GTPγS binding was determined by inclusion of agonists, epinephrine or UK 14304, at 1 or 100 μM. Basal [^{35}S]GTPγS binding was determined in the absence of agonist, and non-specific [^{35}S]GTPγS binding was determined in the presence of unlabeled GTPγS 10 μM. All drugs and compounds, unless specified, were provided by Sigma–Aldrich, Spain. The addition of the α_2-adrenoceptor antagonist RX 821002 was used to demonstrate the specificity of these agonists induced [^{35}S]GTPγS binding values. Autoradiograms were generated by exposing tissue sections to ^{35}S sensitive films (Hyperfilm, Amersham-Pharmacia, UK) for five days, together with ^{14}C microscales (Amersham-Pharmacia, UK). The autoradiograms were analyzed and quantified using image analysis software (Scion Corp., Maryland, USA). Densitometric readings were converted into values of radioligand bound to tissue and expressed as nCi/g tissue.

Previous studies have revealed that brain sections generate sufficient amounts of adenosine under basal conditions, resulting in tonic and widespread A1 receptor-dependent G protein activity (Laitinen, 1999; Moore et al., 2000). In an attempt to