Binding Tests in Respiratory System

Kristy D. Bruse

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Histamine (H_1) Receptor Binding

Purpose and Rationale

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H_1 receptor couples mainly to $G_{q/11}$, thereby stimulating phospholipase C, whereas the H_2 receptor interacts with G_s to activate adenylyl cyclase. The histamine H_3 and H_4 receptors couple to G_i proteins to inhibit adenylyl cyclase and to stimulate MAPK (Hough 2001).

Histamine is considered to play a major role in asthmatic attacks (Bryce et al. 2006). H_1 antagonists have been used since decades as therapeutic agents. This assay is used to determine the affinity of test compounds to the histamine H_1 receptor by measuring their inhibitory activities on the binding of the H_1 antagonist ^{3}H-pyrilamine to a plasma membrane preparation from guinea pig brain.

Procedure

Brains from guinea pigs are homogenized in ice-cold Tris buffer (pH 7.5) in a Potter homogenizer (1 g brain in 30 ml buffer). The homogenate is centrifuged at 4 °C for 10 min at 50,000 g. The supernatant is discarded, the pellet resuspended in buffer, centrifuged as before, and the final pellets

K.D. Bruse (✉)
Integrated Physiology and Pharmacology Consulting, LLC, Poughkeepsie, NY, USA
e-mail: kristy.bruse@gmail.com

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resupended in Tris buffer (1 g fresh weight/5 ml). Aliquots of 1 ml are frozen at −70 °C.

In the competition experiment, 50 µl ^{3}H-pyrilamine (one constant concentration of 2×10^{-9} M), 50 µl test compound (>10 concentrations, 10^{-5}–10^{-10} M) and 100 µl membrane suspension from guinea pig whole brain (approx. 10 mg wet weight/ml) per sample are incubated in a shaking bath at 25 °C for 30 min. Incubation buffer: 50 mM Tris–HCl buffer, pH 7.5.

Saturation experiments are performed with 11 concentrations of ^{3}H-pyrilamine ($0.1–50 \times 10^{-9}$ M). Total binding is determined in the presence of incubation buffer; nonspecific binding is determined in the presence of mepyramine or doxepin (10^{-5} M).

The reaction is stopped by rapid vacuum filtration through glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free radioactivity. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

Evaluation of Results

The following parameters are calculated:

- Total binding of ^{3}H-pyrilamine
- Nonspecific binding: binding of ^{3}H-pyrilamine in the presence of mepyramine or doxepin
- Specific binding = total binding–nonspecific binding
- % inhibition of ^{3}H-pyrilamine binding: 100–specific binding as percentage of control value

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^{3}H-pyrilamine versus nonlabeled drug by a computer-supported analysis of the binding data.

Modifications of the Method

De Backer et al. (1993) reported genomic cloning, heterologous expression in COS-7 cells, and pharmacological characterization of a human H_1-receptor.

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Muscarinic Receptor Binding

Purpose and Rationale

Muscarinic receptors in the airways are important in both the normal physiology and the pathophysiology of pulmonary function. Acetylcholine

released from parasympathetic nerve terminals causes contraction of airway smooth muscle. Animals with asthma or other chronic inflammation of the airways exhibit hypersensitivity of the airways to muscarinic agonists, and muscarinic antagonists are used therapeutically in patients with asthma and chronic obstructive pulmonary disease (Nathanson 2000). Muscarinic receptors are present in neurons in the central and peripheral nervous system, cardiac and smooth muscles, and a variety of exocrine glands. Mammals possess genes encoding five different subtypes of mAChR, termed M_1–M_5, which can be divided into two broad functional categories: the M_1, M_3, and M_5 receptors preferentially couple to the Gq family of G-proteins whereas the M_2 and M_4 receptors preferentially couple to the Gi family of G-proteins.

For involvement of acetylcholine receptors in the gastrointestinal tract see chapter "▶ Pharmacological Effects on Gastric Function".

Several papers deal with the distribution of muscarinic receptors in the lung, their role in pulmonary disease, and the use of muscarinic antagonists for the treatment of obstructive airway disease (Mak and Barnes 1990; Barnes 1993, 2001, 2004; Disse 2001; Disse et al. 1993, 1999; Haddad et al. 1994; Barnes et al. 1995, 1997; Patel et al. 1995; Peták et al. 1996; Alabaster 1997; Matsumoto 1997; Chelala et al. 1998; Hislop et al. 1998; Okazawa et al. 1998; Wale et al. 1999; Rees 2002; Sarria et al. 2002; Tohda et al. 2002; Costello et al. 2006).

Hirose et al. (2001) described the pharmacological properties of a muscarinic antagonist with M_2-sparing antagonistic activity.

Procedure

Binding Affinity for Human and Rat Muscarinic Receptor Subtypes

In competition studies, specific binding of [^{3}H]*N*-methylscopolamine (NMS; New England Nuclear, Boston, Mass., USA) was determined using membranes from Chinese hamster ovary (CHO) cells expressing cloned human m1, m2, m3, m4, or m5 receptors (Receptor Biology, Baltimore, Md., USA), rat m1 or m3 receptors (American Type Culture Collection, Manassas, Va., USA), and rat heart tissue. These CHO cells expressing cloned rat m1 or m3 receptors and rat heart tissue were homogenized in 3 vols of 50 mM Tris–HCl (pH 7.4) and 1 mM EDTA containing 20 % sucrose with a Polytron PT-10. The homogenates were centrifuged at 10,000 g for 30 min at 4 °C. The supernatants were centrifuged at 100,000 g for 60 min at 4 °C. The pellets were suspended in 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ and centrifuged at 100,000 g for 60 min at 4 °C. The pellets were resuspended in the abovementioned buffer (25 mg/ml for CHO cells expressing cloned rat m1 or m3 and 50 mg/ml for rat heart tissue) and stored at −80 °C as membrane preparations. In the binding assay, the membrane preparations were incubated with 0.19 to 0.2 nM [^{3}H]NMS in 50 mM Tris–HCl, 10 mM $MgCl_2$, and 1 mM EDTA (pH 7.4) for 2 h at room temperature. Final protein concentrations were 22 µg/ml (human m1), 70 µg/ml (human m2), 54 µg/ml (human m3), 20 µg/ml (human m4), 116 µg/ml (human m5), 481 µg/ml (rat m1 and m3), and 2500 µg/ml (rat heart). Assays were performed in a total volume of 500 µl. Nonspecific binding was measured in the presence of 1 µM NMS and was less than 2 % of total binding. Free and membrane-bound [^{3}H]NMS were separated by filtration over glass filters (UniFilter-GF/C; Packard Instruments, Meriden, Conn., USA) using a cell harvester (Filtermate 196; Packard Instruments). Radioactivity was counted by a liquid scintillation counter (TopCount; Packard Instruments).

Evaluation

For saturation studies, membranes from CHO cells expressing human m3 were incubated with an increased concentration of [^{3}H]NMS (0.1–3.2 nM) in the presence or absence of 10 nM compound A, and specific binding of [^{3}H]NMS was determined after incubation for 2 h.

Competition binding data were analyzed by a nonlinear regression fitting program using

GraphPad Prism Software (San Diego, Calif., USA). Saturation binding data were transformed to make a Scatchard plot and analyzed by a linear regression fitting program using GraphPad Prism Software.

The K_i values were calculated from the IC_{50} values by using the following equation:

$$K_i = IC_{50}/(1 + [L]/K_d)$$

where K_d is the dissociation constant of [^{3}H]NMS in each receptor subtype and $[L]$ is the concentration of [^{3}H]NMS (Cheng and Prusoff 1973). K_d values of [^{3}H]NMS in each receptor subtype were determined by Scatchard plot analysis. The K_d and B_{max} values below were used in this study. Data of human cloned receptors were extracted from Receptor Biology's Product Information Sheets (Receptor Biology).

Human m1 receptor	K_d = 51 pM	B_{max} = 1.28 pmol/mg of protein
Human m2 receptor	K_d = 290 pM	B_{max} = 1 pmol/mg of protein
Human m3 receptor	K_d = 86 pM	B_{max} = 0.65 pmol/mg of protein
Human m4 receptor	K_d = 56 pM	B_{max} = 1.44 pmol/mg of protein
Human m5 receptor	K_d = 200 pM	B_{max} = 0.59 pmol/mg of protein
Rat m1 receptor	K_d = 62 pM	B_{max} = 0.039 pmol/mg of wet weight
Rat m2 receptor	K_d = 210 pM	B_{max} = 0.0090 pmol/mg of wet weight
Rat m3 receptor	K_d = 72pM	B_{max} = 0.019 pmol/mg of wet weight

In saturation studies, the K_i value was calculated using the following equation:

$$K_i = K_d/\left(K'_d - K_d\right) \times [C]$$

where K'_d or K_d is the dissociation constant of [^{3}H]NMS in human m3 receptors in the presence or absence of an inhibitor, respectively, and $[C]$ is the concentration of the test drug (Nishikibe et al. 1999).

Modifications of the Method

Struckmann et al. (2003) investigated the role of muscarinic receptor subtypes in the constriction of peripheral airways by studies on receptor-deficient mice.

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