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Labeling of rat heart muscarinic receptors using the new M_2 selective antagonist [^{3}H]AF-DX 384

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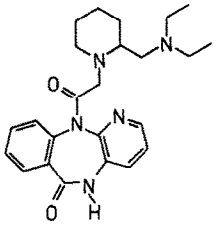
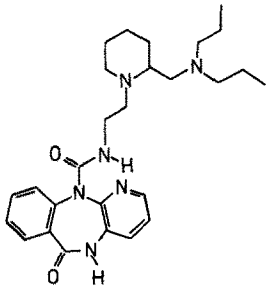
There is now clear experimental evidence supporting the existence of muscarinic acetylcholine receptor subtypes (M_1, M_2, M_3) [1–3]. M_1 receptors are mainly found in neuronal tissues and possess a high affinity for pirenzepine [4]. Muscarinic receptors in cardiac and glandular tissues can be distinguished by the use of selective antagonists such as AF-DX 116 (11-2((-((diethylamino)methyl)-1-piperidinyl)-acetyl)-5,11-dihydro-6*H*-pyrido(2,3-b) (1,4)-benzodiazepin-6-one) which is cardioselective [5] and 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) or hexahydro-sila-difenidol (HHSiD) which are selective for glandular preparations [6, 7]. It has been suggested to classify those receptors with high affinity for AF-DX 116 as M_2 and those with high affinity for 4-DAMP and HHSiD as M_3 [2].

Recently Eberlein and colleagues described the development of a potent and highly selective antagonist of the cardiac M_2 muscarinic receptor subtype, AF-DX 384 (5,11-dihydro-11-(((2-(2-((dipropylamino)methyl)-1-piperidinyl)ethyl)amino)carbonyl)-6*H*-pyrido(2,3-b) (1,4)-benzodiazepin-6-one methansulfonate) [8]. In *in vitro* binding studies AF-DX 384 shows a 16-fold higher affinity for cardiac M_2 than for M_3 muscarinic receptors in the salivary gland.

We report here for the first time on the binding of [^{3}H]AF-DX 384 to membranes from rat heart. Competition of [^{3}H]AF-DX 384 binding was investigated in receptor binding studies using the muscarinic antagonists atropine, AF-DX 116, AF-DX 384, 4-DAMP, hexahydro-sila-difenidol and pirenzepine.



AF-DX 116



AF-DX 384

Material and Methods

Chemicals. [^{3}H]AF-DX 384 (3.33 TBq/mmol) and [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB; 1.22 TBq/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). 4-DAMP was purchased from Biotrend GmbH (Cologne, F.R.G.). Racemic AF-DX 116 and AF-DX 384 as well as hexahydro-sila-difenidol and pirenzepine were synthesized at Dr Karl Thomae GmbH (Biberach, F.R.G.). All other chemicals were of best grade available.

Binding experiments. Rat heart homogenates were prepared as follows: Male Wistar (Chbb:THOM strain, 180–

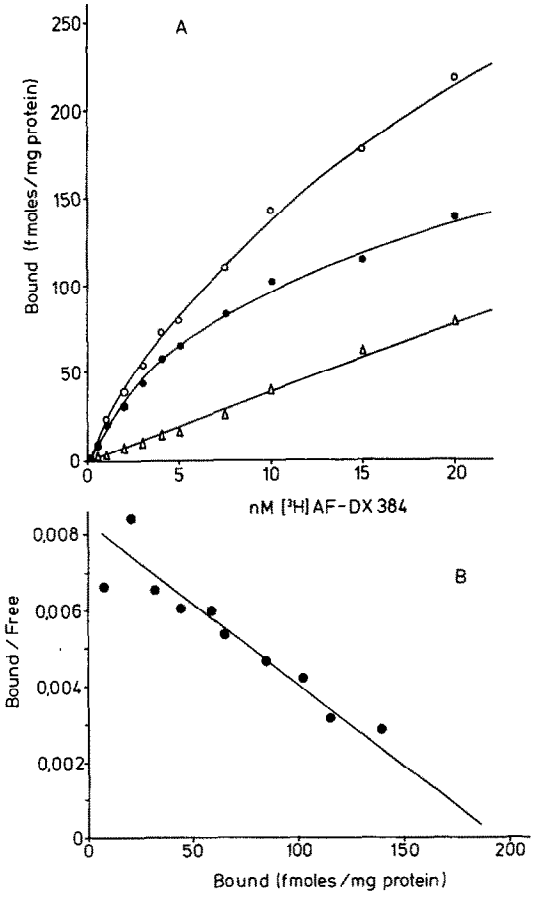


Fig. 1(A). Saturation curve of [^{3}H]AF-DX 384 binding to rat heart homogenates. (○) Total, (●) specific and (△) non-specific binding of [^{3}H]AF-DX 384 were determined for 60 min at 21°. Values are the means of a typical experiment done in triplicate. Non-specific binding was determined in the presence of 1 μM (−)-quinuclidinyl benzilate. (B): Scatchard plot of saturation data.

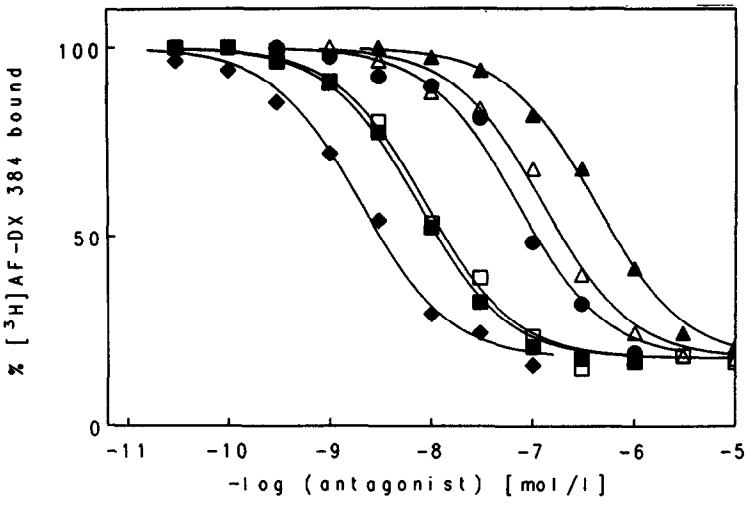


Fig. 2. Displacement of [^{3}H]AF-DX 384 by the muscarinic antagonists atropine (◆), AF-DX 116 (●), AF-DX 384 (■), 4-DAMP (□), HHSiD (△) and pirenzepine (▲). The data points are the means of three independent experiments for each compound and were analysed with the TOPFIT program.

200 g) rats were killed by a blow on the neck. The heart was dissected out, weighed and homogenized in HEPES buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl; pH 7.5) by an Ultra-Turrax at maximal setting for 60 sec. The homogenate was diluted 400 times regarding the original tissue weight. Protein (0.4 mg) was incubated in HEPES buffer with 0.1–20 nM [^{3}H]AF-DX 384 or 5–500 pM [^{3}H]QNB for saturation or 2 nM [^{3}H]AF-DX 384 for displacement studies at 21° for 60 min in a total volume of 1 mL. Particle bound radioligand was assayed by liquid scintillation counting after rapid filtration through polyethyleneimine treated GF/B glass fibre filters. Non-specific binding was defined as radioactivity bound in the presence of 10^{-6} M (−)-quinuclidinyl benzilate. Binding data were analysed by a computer assisted non-linear least-square curve fitting method, TOPFIT [9], IC_{50} values were converted to K_i values according to Cheng and Prusoff [10]. The protein content was determined by the method of Lowry *et al.* [11]. Bovine serum albumin was used as standard.

Results

Binding of [^{3}H]AF-DX 384 (2 nM) to muscarinic receptors in rat increased linearly with tissue concentration used (0.1–1.0 mg protein/assay tube). Figure 1 shows the binding of [^{3}H]AF-DX 384 to rat heart homogenates. The saturation isotherm for [^{3}H]AF-DX 384 was best defined by the interaction of the radioligand with a single population of saturable receptor sites with an equilibrium dissociation constant (K_D) of 8.7 ± 0.6 nM (± SD, N = 3). The corresponding receptor density (B_{max}) was 215 ± 30 fmol/mg protein. Non-specific binding did not exceed 28% of total radioactivity bound at the K_D of [^{3}H]AF-DX 384. In comparative studies using [^{3}H]QNB a receptor density of 179 ± 21 fmol/mg protein was determined. The dissociation constant of this radioligand was 81 ± 11 pM.

The time course of specific [^{3}H]AF-DX 384 was determined in kinetic experiments. The $T_{1/2}$ of association of [^{3}H]AF-DX 384 (2 nM) was approximately 8.2 min and equilibrium was reached after 45 min of incubation (data not shown). The $T_{1/2}$ of dissociation for [^{3}H]AF-DX 384 from these sites was 10 min. The rate constants for association (K_{+1}) and dissociation (K_{-1}) were $6.14 \times 10^6 \pm 2.49 \times 10^{-6}$ M^{-1} min^{-1} (± SD, N = 3) and 0.0688 ± 0.0036 min^{-1} respectively. The ratio K_{-1}/K_{+1} of the rate constants was 11.2 ± 4.5 nM and provides an independent rough estimate of the equilibrium constant K_D.

Specific binding of [^{3}H]AF-DX 384 was inhibited by the non-selective antagonist atropine (K_i: 1.55 ± 0.14 nM), by the M_1 selective antagonist pirenzepine (K_i: 436 ± 49 nM), by the M_2 selective antagonists AF-DX 116 (K_i: 53.8 ± 4.6 nM) and AF-DX 384 (K_i: 6.8 ± 1.8 nM) as well as by 4-DAMP (K_i: 7.1 ± 1.2 nM) and HHSiD (K_i: 124 ± 2 nM) (Fig. 2). The Hill coefficients for all competition curves were not significantly different from unity.

Discussion

In the present study [^{3}H]AF-DX 384 has been used to label muscarinic receptors in the rat heart. The dissociation constants of [^{3}H]AF-DX 384, determined from either saturation experiments or kinetic studies, are in good agreement with previously reported data obtained from indirect studies with unlabeled AF-DX 384 [8]. The density of labeled binding sites in our preparations is higher than reported for [^{3}H]QNB and [^{3}H]AF-DX 116 [12]. This discrepancy might be explained by different methods of tissue preparations since comparative studies using [^{3}H]QNB in our preparations revealed no significant differences in receptor number.

Specific binding of [^{3}H]AF-DX 384 was inhibited by muscarinic antagonists. The rank order of potencies for these antagonists was atropine > AF-DX 384 > 4-DAMP > AF-DX 116 > HHSiD > pirenzepine. The Hill coefficients of all displacement curves were not significantly different from unity indicating binding of [^{3}H]AF-DX 384 to an homogenous population of muscarinic receptor sites. The inhibition constants and the affinity pattern of the antagonists is consistent with the labeling of cardiac M_2 muscarinic receptors by [^{3}H]AF-DX 384 [13].

In conclusion, the selective antagonist radioligand [^{3}H]AF-DX 384 can be successfully used to label and study M_2 muscarinic receptors. The use of this new stable and selective antagonist radioligand will facilitate M_2 receptor research.

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