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HIGH-AFFINITY [^{3}H]PIRENZEPINE BINDING TO PUTATIVE M_1 MUSCARINIC SITES IN THE NEUROBLASTOMA x GLIOMA HYBRID CELL LINE (NG 108-15)

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The specific binding of both the non-classical antagonist [^{3}H] pirenzepine ([^{3}H]PZ) and the classical antagonist [^{3}H](-)quinuclidinyl benzilate ([^{3}H](-)QNB) was determined in parallel assays of the mouse neuroblastoma x rat glioma hybrid cell line (NG 108-15). Saturation isotherms yielded a K_d = 4.0 nM and Bmax = 27.8 fmoles/mg protein for [^{3}H]PZ and a K_d = 17.2 pM and Bmax = 53.2 fmoles/mg protein for [^{3}H](-)QNB. The inhibition data of pirenzepine vs [^{3}H](-)QNB was best fit to a 2-site binding model revealing both a high affinity pirenzepine site (72%, K_H = 10.3 nM) and a low affinity site (28%, K_L = 97.5 nM). [^{3}H]PZ competition studies demonstrated stereospecificity, steep inhibition curves for muscarinic antagonists (Hill coefficients close to 1), and a shallow inhibition curve for a muscarinic agonist. These results indicate that muscarinic receptors on NG 108-15 cells may be subclassified (M_1/M_2) on the basis of the discriminative capability of [^{3}H]PZ.

Recent studies of the muscarinic receptor have provided evidence for the existence of receptor subtypes (1-11), based primarily upon the unique pharmacological profile of pirenzepine, a novel muscarinic antagonist (2-11). Pirenzepine displays a large variation in affinity in indirect binding studies of different tissues: High affinities (Ki values of 20-50 nM) were obtained in the sympathetic ganglia and cerebral cortex, while lower affinities (Ki value of 800 nM) were seen in the ileum and myocardium (2,4,8). Moreover, though these latter tissues obey the law of mass action, the former tissues do not. Physiological data also show that pirenzepine is more potent on the superior cervical ganglia than on the ileum (3). Based upon these observations, it has been suggested that pirenzepine is capable of discriminating between different muscarinic receptor subtypes. Adopting

Abbreviations Used: K_H, K_d value of high affinity site; K_L, K_d value of low affinity site; $IC_{50(H)}$, IC_{50} value of high affinity site; $IC_{50(L)}$, IC_{50} value of low affinity site.

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the terminology of Goyal and Rattan (1), we have referred to the sites which [^{3}H]PZ binds with high affinity as M_1.

In support of a M_1/M_2 concept, a series of recent investigations in our laboratory have clearly demonstrated that high affinity [^{3}H]PZ binding sites are predominantly distributed in certain discrete rat brain regions (5-7,10) and human stellate ganglia (9,10) as judged by the comparison with the relative densities of [^{3}H](-)QNB binding sites in these same structures. We now report that [^{3}H]PZ binds with high affinity to a subpopulation of muscarinic receptor binding sites in the mouse neuroblastoma x rat glioma hybrid cell line (NG 108-15) (12-15) and have characterized these binding sites.

Materials and Methods

NG 108-15 cells (a mouse neuroblastoma x rat glioma hybrid) (16) were generously donated by Dr. A. Blume of the Roche Institute of Molecular Biology (Nutley, NJ). These cells of low passage were grown in tissue culture flasks obtained from Costar (Cambridge, MA) in an atmosphere of humidified air (90%)/CO_2 (10%) at 37°C. The growth medium consisted of 90% Dulbecco's modified Eagle's medium (DMEM) obtained from Gibco Laboratories (Santa Clara, CA) and 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA) supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml, (Eli Lilly and Co., Indianapolis, IN), hypoxanthine (100 µM), aminopterin (1 µM) and thymidine (16 µM). Cells were subcultured routinely with Trypsin-EDTA solution (Trypsin, 0.25%; EDTA, 0.02%, Gibco Laboratories). When the cells reached confluency, they were harvested in the following manner: After rapid shaking of the flasks, the cells were separated from the growth medium by centrifugation at 1000 g for 5 min, with the resulting pellet being washed three times with Dulbecco's phosphate buffered saline (NaCl, 143 mM; KCl, 2.8 mM; $CaCl_2$, 0.96 mM; $MgCl_2$, 0.51 mM; Na_2HPO_4, 6.8 mM; KH_2PO_4, 1.2 mM adjusted to pH 7.4) by centrifugations at 1000 g for 2 min. Cell number was determined with a hemocytometer. Subsequently, the cells were rewashed with 10 mM $NaKPO_4$ buffer (Na_2HPO_4, 8.1 mM; KH_2PO_4, 1.9 mM, pH 7.5). The pelleted intact cells were rapidly frozen in liquid nitrogen and stored at -80°C until assay.

To make cell membranes for the muscarinic receptor binding assays, the frozen cells were thawed and suspended in 10 mM $NaKPO_4$ buffer. The cell homogenate was prepared by homogenizing with a Brinkmann® polytron (at setting 4, 3 x 15 sec. bursts) and centrifuged at 48,000 g for 15 min. The resulting pellet was resuspended in the same buffer and rehomogenized using the polytron. Under microscopic examination of the homogenate, all the cells were ruptured into fine membrane particles.

Specific [^{3}H]PZ (84.0 Ci/mmole, New England Nuclear (NEN), MA) binding was determined using the previously described filtration assay of Watson et al (6) with a slight modification. Briefly, [^{3}H]PZ (1.6 - 21.0 nM) was incubated with the cell homogenate ($\simeq 10^6$ cells) in final volume of 1.0 ml of 10 mM $NaKPO_4$ buffer at 25°C for 90 min unless otherwise indicated. The final concentration of protein per assay tube (1 ml) was 0.3 - 0.4 mg. Nonspecific [^{3}H]PZ binding was determined in the presence of 10 µM atropine sulfate and all assays were performed in triplicate. The

reaction was terminated by rapid filtration through Whatman glass fiber filters (GF/B) which were presoaked in 0.1% polyethylenimine (PEI, Sigma Co., St. Lois, MO) for 6 hours prior to use. The filters were rinsed three times with 5 ml of ice-cold 10 mM $NaKPO_4$ buffer to remove excess free [^{3}H]PZ.

Specific [^{3}H](-)QNB (33.1 Ci/mmole, NEN, MA) binding was performed as previously described by Yamamura and Snyder (17) with a slight modification. [^{3}H](-)QNB (7.2 - 780 pM) was incubated with NG 108-15 cell homogenates ($\simeq 10^6$ cells) which were prepared as described above in final volume of 2.0 ml in 10 mM $NaKPO_4$ buffer at 37°C for 90 min unless otherwise indicated. 1 μM atropine sulfate was used to determine nonspecific binding. Each binding assay was performed 4 separate times in duplicate.

For both ligands radioactivity trapped on the filter was extracted overnight in 6 ml of scintillation cocktail (Omnifluor 16 gm/Triton X-100 1 L/Toluene 2 L) and measured in a Searle Analytic 81 liquid scintillation counter with an efficiency of 41-45%.

Saturation and inhibition data were analyzed using non-linear least squares regression analysis (18) from programs prepared for the Apple II plus microcomputer (S.H.M. Research, Tucson, AZ). The averaged data from independent experiments was weighted (inverse of coefficient of variation) or unweighted to obtain the best fitted curves. Protein was determined according to the method of Lowry et al. (19) using bovine serum albumin as a standard.

Results

The competition curves of atropine and pirenzepine vs [^{3}H](-)QNB are depicted in Fig. 1. The IC_{50} values for atropine and pirenzepine were 0.57 nM and 267 nM, respectively. While the atropine vs [^{3}H](-)QNB competition curve yielded a Hill coefficient of nearly one (0.92), the inhibition of [^{3}H](-)QNB binding by pirenzepine gave rise to a Hill coefficient of 0.86. The computer-assisted analyses of one-site and two-site fit were compared for both the atropine and pirenzepine inhibition of [^{3}H](-)QNB by using a partial F-test (20). There was no statistically significant difference between the data analyses of the one-site and two-site fit for atropine. However, for the pirenzepine vs [^{3}H](-)QNB competition curve, the two-site fit analyses yielded a significant resolution as compared to the one-site fit ($P < 0.001$), revealing that pirenzepine has a high affinity site (72%, $IC_{50(H)}$ = 149 nM or K_H = 10.3 nM) and a low affinity site (28%, $IC_{50(L)}$ = 1413 nM or K_L = 97.5 nM).

Specific [^{3}H]PZ (at 4 nM) binding showed a linear relationship with protein concentration up to 0.8 mg protein ($\simeq 2 \times 10^6$ cells)/assay tube (data not shown). Therefore, a homogenate of 10^6 cells was routinely used in all subsequent binding assays. Typically, 40-50% specific binding was

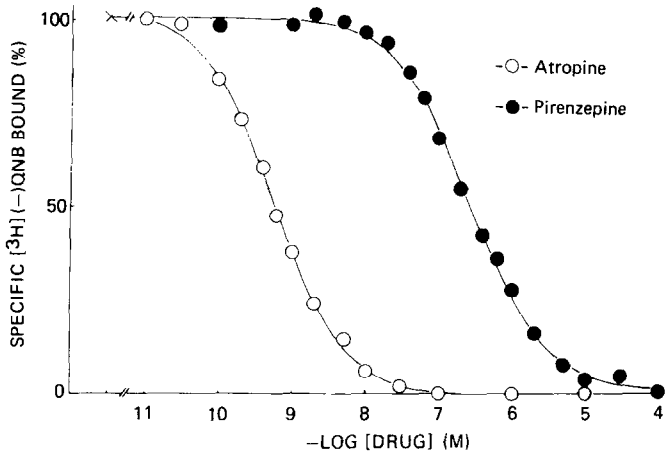

Figure 1. Inhibition of [^{3}H](-)QNB binding by atropine and pirenzepine in NG 108-15 cell homogenates. Various concentrations of the unlabeled drug (○, atropine; ●, pirenzepine) were incubated with 0.23 nM of [^{3}H](-)QNB and cell homogenate (≈ 10^6 cells) for 60 minutes at 25°C in 10 mM $NaKPO_4$ buffer. Each point represents the mean % specific [^{3}H](-)QNB binding from 4 independent experiments carried out in duplicate. Inhibition curves represent the best fit of non-linear least squares regression analyses. Inhibition values for each drug are as follows; 1) atropine, IC_{50} = 0.57 ± 0.04 nM (0.46 - 0.64 nM) and Hill coefficient = 0.92 (0.89 - 0.97), 2) pirenzepine, IC_{50} = 267 ± 28 nM (204 - 321 nM) and Hill coefficient = 0.86 (0.82 - 0.90). The computer - assisted two-site best fit analysis revealed that pirenzepine displaceable [^{3}H](-)QNB specific binding sites consist of a high affinity site (72%, K_H = 10.3 nM) and a low affinity site (28%, K_L = 97.5 nM). The two-site fit analyses yielded a significant resolution as compared to the one-site fit ($p < 0.001$, partial F-test).

obtained at a concentration of 2 nM [^{3}H]PZ. Fig. 2A and 2B illustrate computer-assisted best fits of parallel saturation isotherms of specific [^{3}H](-)QNB and [^{3}H]PZ binding, respectively. An apparent dissociation constant (K_d) = 17.2 pM (15.0 - 19.2 pM) and a maximal number of binding sites (Bmax) = 53.2 fmoles/mg protein (50.4 - 56.2 fmoles/mg protein) were obtained for [^{3}H](-)QNB. The saturation isotherm for [^{3}H]PZ yielded a K_d value of 4.0 nM (2.2 - 12.0 nM) and a Bmax value of 27.8 fmoles/mg protein (18.4 - 43.6 fmoles/mg protein), indicating that [^{3}H]PZ labels over 50% of these sites labeled by [^{3}H](-)QNB.

Several muscarinic drugs were tested for their relative potencies in inhibiting the specific binding of [^{3}H]PZ (at 6 nM) (Fig. 3). All of the muscarinic antagonists tested displayed Hill coefficients which were close to 1.0. Atropine was about 300 fold more potent than pirenzepine. Stereospecificity was clearly demonstrated by the 5000 fold differences in

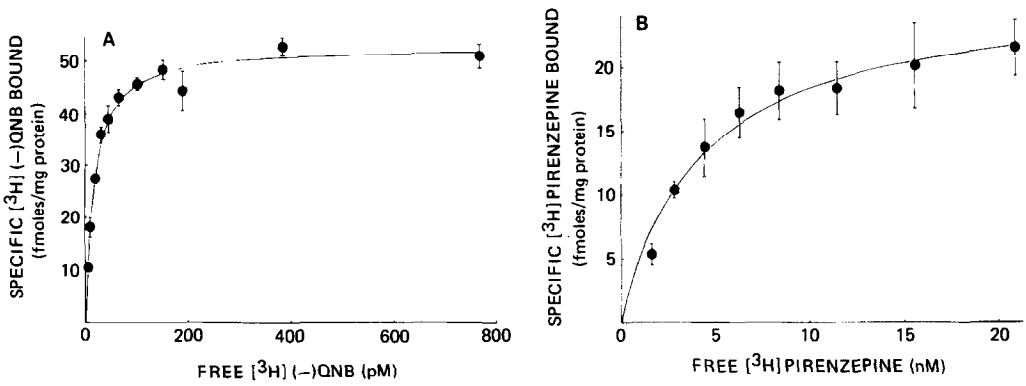

Figure 2. Saturation isotherms of [^{3}H](-)QNB and [^{3}H]PZ binding to NG 108-15 cell homogenates. The concentration ranges for the saturation isotherms were 7.2 - 780 pM and 1.6 - 21.0 nM for [^{3}H](-)QNB and [^{3}H]PZ, respectively. Each point represents the mean (± S.E.M.) of specific [^{3}H](-)QNB bound (Fig. 2A.) at 37°C for 90 min or [^{3}H]PZ (Fig. 2B) bound at 25°C for 90 min in 4 independent but parallel experiments performed in duplicate ([^{3}H](-)QNB) or in triplicate ([^{3}H]PZ). Curves represent the weighted best fitted non-linear least squares regression analyses of the data. K_d values (± S.E.M.) calculated as the geometric mean of the individual data are 17.2 ± 0.9 pM (15.0 - 19.2 pM) and 4.0 ± 1.5 nM (2.2 - 12.0 nM) for [^{3}H](-)QNB and [^{3}H]PZ, respectively. Bmax values (± S.E.M.) calculated as the arithmetic mean of the individual data are 53.2 ± 1.4 fmoles/mg protein (50.4 - 56.2 fmoles/mg protein) and 27.8 ± 5.6 fmoles/mg protein (18.4 - 43.6 fmoles/mg protein) for [^{3}H](-)QNB and [^{3}H]PZ, respectively. The highest [^{3}H]PZ concentration used in the binding assay bound about 84% of the M_1 receptors. Higher concentrations of [^{3}H]PZ were not used since they yielded a large portion of nonspecific binding.

the potencies of the enantiomers dexetimide and levetimide in displacing specific [^{3}H]PZ binding. Contrary to the steep binding curves produced by the muscarinic antagonists, the potent agonist oxotremorine produced a shallow binding curve (Hill coefficient = 0.43), indicating multiple affinity agonist states for the high affinity M_1 site as labeled with [^{3}H]PZ.

Discussion

The mouse neuroblastoma x rat glioma hybrid cell line (NG 108-15) has been shown to possess several cholinergic markers including choline acetyltransferase (16) and muscarinic cholinergic receptors (12-15). The potential usefulness of this cell line as a model of neural cells in the central nervous system has been well documented in a series of neurotransmitter receptor studies (12-15,21,22). The number of [^{3}H](-)QNB binding sites in NG 108-15 cell homogenates in our studies is consistent

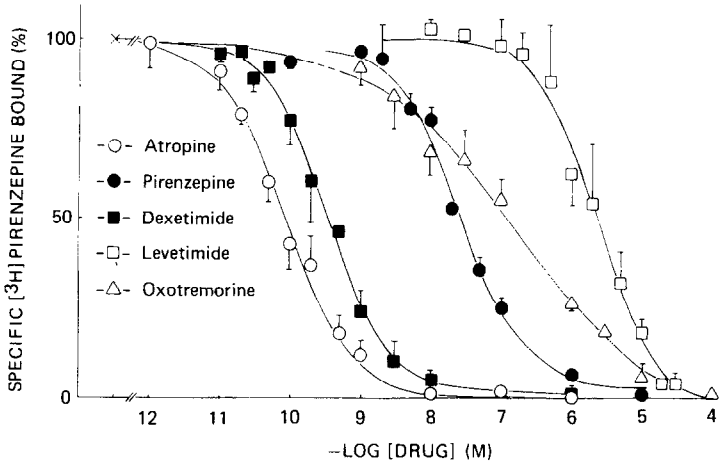

Figure 3. Inhibition of [^{3}H]PZ binding by muscarinic cholinergic drugs in NG 108-15 cell homogenates. Various concentrations of unlabeled drugs (○, atropine; ●, pirenzepine; ■, dexetimide; □, levetimide; △, oxotremorine) were incubated with 6 nM of [^{3}H]PZ for 90 min at 25°C. Each point represents the mean (± S.E.M.) specific [^{3}H]PZ binding (%) obtained from 4 independent experiments carried out in triplicate. Inhibition curves represent the best fit of non-linear least squares regression analyses. Inhibition values for each drug are as follows; 1) atropine, IC_{50} = 0.10 ± 0.02 nM (0.05 - 0.14 nM) and Hill coefficient = 0.92 (0.79 - 1.00), 2) pirenzepine, IC_{50} = 26.1 ± 5.1 nM (15.7 - 35.5 nM) and Hill coefficient = 1.00 (0.98 - 1.27); 3) dexetimide, IC_{50} = 0.33 ± 0.08 nM (0.17 - 0.51 nM) and Hill coefficient = 1.02 (0.81 - 1.45); 4) levetimide, IC_{50} = 1566 ± 865 nM (373 - 4745 nM) and Hill coefficient = 1.03 (1.02 - 1.57); 5) oxotremorine, IC_{50} = 170 ± 84 nM (68 - 444 nM) and Hill coefficient = 0.43 (0.32 - 0.57).

with previously reported values (12,13). The K_d value (17.2 pM) of [^{3}H](-)QNB binding appears of higher affinity than previously reported value of 100 pM (12,13). This is probably due to the use of higher specific activity and the active isomer [^{3}H](-)QNB.

The nonclassical muscarinic antagonist pirenzepine vs [^{3}H](-)QNB competition curve yielded an IC_{50} value = 267 nM, which can be converted into a Ki value of 18.4 nM by using the Cheng & Prusoff (23) equation, $Ki = IC_{50}/(1+[L]/K_d)$, in which [L] is the concentration of [^{3}H](-)QNB (0.23 nM) and K_d is 17.2 pM for [^{3}H](-)QNB. This Ki value is in good agreement with the high affinity pirenzepine sites as described previously (2,4,8). Moreover, the Hill coefficient (0.86) indicates a deviation from the law of mass action, and the two-site fit reveals that 72% of pirenzepine displaceable [^{3}H](-)QNB sites are of high affinity (K_H = 10.3 nM).

Our direct [^{3}H]PZ binding studies verify that this radioligand binds with high affinity to NG 108-15 cell homogenates. The K_d value of 4.0 nM derived from the non-linear least squares regression analyses of the saturation isotherms are compatible with those obtained for [^{3}H]PZ binding in the rat cerebral cortex, corpus striatum, hippocampus and human stellate ganglia in previous studies (6,9,10). In good agreement with the high affinity pirenzepine site observed from the indirect competition study, the Bmax value for specific [^{3}H]PZ binding indicates that a substantial proportion (52%) of the total muscarinic receptor density (Bmax of [^{3}H](-)QNB) are of the putative M_1 subtype. The value of 52% is probably an underestimate of the number of M_1 sites, because we used concentrations of [^{3}H]PZ up to 21 nM which labels only 84% of the M_1 sites. Use of higher concentrations of [^{3}H]PZ was difficult due to the presence of high nonspecific binding. Thus, both direct and indirect binding studies suggest that NG 108-15 cells possess both of the muscarinic receptor subtypes (M_1/M_2), but a predominance of the M_1 subtype.

These studies verify and extend our previous work regarding the subclassification of muscarinic chilinergic receptors based upon the discriminative properties of pirenzepine. M_1 sites labeled with [^{3}H]PZ have been localized primarily to neural tissues (cerebral cortex, hippocampus and stellate ganglia) (6,9,10) and [^{3}H]PZ binding has been shown to be modulated by ions but not by guanine nucleotides. The functional significance of the M_1 site on NG 108-15 cells is not yet known. However this cell line has been shown to possess muscarinic receptor-mediated stimulation of phosphatidyl inositol turnover (15,24) which has been suggested as a possible effector for the putative M_1 site (11). Additionally, there are also some functional aspects which may be associated with the M_2 sites: 1) Muscarinic receptor activity is inversely coupled with adenylate cyclase (21,22) and 2) 5-guanylylimidodiphosphate produces a decrease in the affinity of carbamylcholine for competing with [^{3}H](-)QNB binding to NG 108-15 cells

(14). This effect is notable (10-15 fold) in the myocardium (mostly M_2 receptor subtype), but is minimally observed in the cerebral cortex (mostly M_1 receptor subtype) (11). The aforementioned different forms of receptor - effector transduction in NG 108-15 cells may be attributed to different muscarinic receptor subtypes in this cell line.

In conclusion, we have shown specific high affinity [^{3}H]PZ binding to NG 108-15 cell homogenates. In addition, we have presented evidence suggesting that 50-70% of the muscarinic receptors are of the M_1 subtype in this cell line. Further studies of regulatory mechanisms, receptor-effector coupling and turnover of putative M_1 and M_2 receptor subtypes should promote a greater understanding of the role of each receptor subtype in cholinergic neurotransmission. While this demonstration of antagonist heterogeneity in a neural cell line in culture provides further evidence in support of the M_1/M_2 subclassification, a more precise definition regarding the nature of these subtypes is probably dependent upon the eventual determination as to whether these sites are the result of primary structural differences or differential coupling mechanisms.

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be also a useful tool for determining the distribution of other metals (M), including iron, by preparing the mixed complex of $M_nCu_{2-n}Tf$.

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