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INHIBITION OF PHOSPHOINOSITIDE TURNOVER BY SELECTIVE MUSCARINIC ANTAGONISTS IN THE RAT STRIATUM

CORRELATION WITH RECEPTOR OCCUPANCY*

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Abstract—In the rat corpus striatum, receptor occupancy and the inhibition of phosphoinositide turnover by muscarinic antagonists have been examined under very similar conditions with respect to tissue preparation and buffer composition. The results suggest a good correlation between receptor occupancy and inhibition by muscarinic antagonists, of the carbachol-stimulated turnover of inositol phospholipids, measured by the accumulation of [^{3}H]inositol phosphates in the presence of 5 mM LiCl. In the presence of 10 mM carbachol (CCh), the accumulation of labeled inositol phosphates was increased 8-fold above basal levels (EC_{50} = 95 μM). Inclusion of antagonists resulted in a dose-dependent inhibition of the 0.1 mM CCh-stimulated inositol phosphate accumulation, with a rank order of potency of atropine > trihexyphenidyl > pirenzepine ≫ gallamine. Radioligand binding studies with [^{3}H]-*l*-quinuclidinyl benzilate ([^{3}H]QNB) in a cell aggregate preparation revealed a single class of saturable, high affinity [^{3}H]QNB binding sites exhibiting a K_d of 74 pM and a B_{max} of 2.85 pmol/mg protein. The antagonists examined were able to inhibit the binding of [^{3}H]QNB with the same rank order of potency as for the inhibition of carbachol-stimulated phosphoinositide turnover (atropine > trihexyphenidyl > pirenzepine ≫ gallamine). Although the inhibition of phosphoinositide turnover and [^{3}H]QNB binding by the nonselective antagonist atropine was best described by interaction at a single site, inhibition of phosphoinositide turnover and [^{3}H]QNB binding by both pirenzepine, which is selective for M1 receptors, and gallamine, which is selective for M2 receptors, is complex. Pirenzepine was much more potent than gallamine for both binding to receptors and inhibiting phosphoinositide turnover. Nonlinear curve-fitting analysis indicated that slope factors for inhibition of phosphoinositide turnover (analogous to Hill coefficient for binding) by only subtype selective antagonists were significantly less than unity. The above-mentioned antagonist interactions together with the apparently multicomponent stimulation of phosphoinositide turnover by carbachol suggest that phosphoinositide turnover may be coupled to more than one muscarinic receptor subtype in the corpus striatum.

The muscarinic acetylcholine receptor exists as a heterogeneous population with a distinct regional distribution in the mammalian CNS [1–3]. On the basis of agonist affinity, Birdsall *et al.* [4] demonstrated three classes of muscarinic receptor, termed super-high (SH), high (H), and low (L). These subtypes are distributed in different proportions throughout the rat brain, with the brainstem (pons, medulla, and cerebellum) possessing predominantly SH and H sites, whereas forebrain structures (cerebral cortex, hippocampus, and corpus striatum) possess primarily L sites [4]. In addition, it was proposed that the L subtype was most likely responsible for the biological responses to muscarinic receptor activation. In contrast, the binding of the non-classical antagonist pirenzepine (PZ) has identified two muscarinic receptor subtypes, commonly termed M1 and M2 [5, 6]. M1 receptors exhibit high affinity for pirenzepine and low affinity for carbachol, whereas M2 receptors exhibit low affinity for pirenzepine and high affinity for carbachol [7]. The M1 and M2 muscarinic receptors are also differentially distributed in the rat brain [2]; however, the ratio of M1 to M2 sites does not always correlate well with observed ratios of SH, H, and L agonist sites (see Ref. 8 for discussion).

Recently, the muscarinic receptor of the mammalian CNS has been shown to be coupled to the stimulation of phosphoinositide turnover [9] via the coupling of the receptor to phospholipase-C, probably through interaction with a regulatory GTP-binding protein [10]. Recent studies have suggested that the M1 subtype is coupled to the stimulation of phosphoinositide turnover, as evidenced by the ability of pirenzepine to inhibit carbachol-stimulated phosphoinositide turnover with a potency which correlates with the high affinity pirenzepine binding site [11]. However, a number of observations suggest

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that the relationship between muscarinic receptor subtypes and biochemical responses is more complex. It has been suggested that stimulation of phosphoinositide turnover may be coupled to more than a single subtype, as evidenced by variations in the ability of pirenzepine to antagonize carbachol-stimulated phosphoinositide turnover in guinea pig brain [12], rat brainstem [13], guinea pig colon and bladder [14], embryonic chick heart and human astrocytoma cells [15].

While previous studies have indicated that phosphoinositide turnover stimulated by muscarinic agonists is sensitive to inhibition by the M1-selective antagonist pirenzepine [11, 12], these studies have not addressed whether or not this inhibition is limited to the M1-selective antagonists. The present study was aimed at examining the abilities of both pirenzepine and the M2-selective antagonist gallamine [16, 17] to inhibit carbachol-stimulated phosphoinositide turnover in the rat corpus striatum. In addition, the receptor occupancy profiles of these ligands have been examined under conditions similar to those used for the assay of phosphoinositide turnover. A good correlation was found between receptor occupancy profiles and inhibition of phosphoinositide turnover.

METHODS

Phosphoinositide turnover. Male Sprague–Dawley rats (200–300 g) were killed by cervical dislocation, and the brains were removed and cooled to 4° in modified Krebs–Hensleit buffer (KHB) (NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 1.3 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; and glucose, 11.7 mM) [9] which had been adjusted to pH 7.4 by gassing with 95% O_2/5% CO_2. The corpus striatum was rapidly dissected out on a cold surface and cross chopped (350 μm × 350 μm) on a McIlwain tissue chopper at 4°. Tissue slices from three rats were suspended in 5 ml KHB and incubated for 1 hr at 37° with buffer changes at 20-min intervals. The slices were then transferred to a conical centrifuge tube and allowed to settle.

Aliquots (25 μl) of striatal slices were then added to 4-ml plastic vials (Beckman Bio-vials) containing 255 μl KHB plus 5 mM LiCl and 0.3 μM [^{3}H]inositol (13–16 Ci/mmol, Amersham) and gassed with 95% O_2/5% CO_2. After 30 min at 37°, 10 μl of buffer or appropriate concentration of agonist was added, and the incubation was continued for 45 min. When antagonists were used, they were added (in 10 μl KHB) 10 min prior to the addition of agonist. The incubation was then continued for 45 min. Incubations were terminated by the addition of 0.94 ml $CHCl_3$/MeOH (1:2, v/v), followed by 0.31 ml $CHCl_3$ and 0.31 ml H_2O, and centrifugation at 1000 *g* for 10 min to separate the phases. Then, 750 μl of the upper (aqueous) phase was removed and diluted to 3 ml with 5 mM myo-inositol. Ion-exchange resin (500 μl) (Dowex-1X8, formate form, Bio-Rad) was added (1:1 wt:vol. slurry), and the resin was washed three times with 3 ml of 5 mM myo-inositol. The labeled inositol phosphates were then eluted by the addition of 0.5 ml of 0.1 M formic acid/1 M ammonium formate, and 450 μl of eluate was removed and counted in 5 ml Liquiscint (National Diagnostics) scintillation fluid. In some experiments labeled inositol phosphates were separated by ion exchange chromatography. The distribution of label was as follows: inositol phosphate (86%), inositol bisphosphate (12%) and inositol triphosphate (2%).

[^{3}H]-l-*Quinuclidinyl benzilate binding in cell aggregates.* Cell aggregates for binding assays were prepared according to methods modified from those of El-Fakahany and Lee [18] as follows. Striatal tissue was cross chopped at 350-μm intervals and incubated for 1 hr at 37° in KHB gassed with 95% O_2/5% CO_2, with two changes of buffer. The slices were collected by gentle centrifugation, weighed, and suspended in minimal volume of ice-cold KHB. The suspended slices were gently forced through tightly stretched nylon mesh (125 μm), and the resulting aggregates recovered in 10 ml KHB. The cell aggregates thus formed are approximately 100 μm in diameter and exhibit 90% viability as determined by dye exclusion. The aggregates were washed twice with 10 ml of cold KHB and resuspended at a final concentration of 20 mg (slice wet weight) per ml. Aliquots (50 μl) of the suspended cell aggregates (5–10 μg total protein) were added to a final volume of 4 ml KHB containing 0.01 to 1 nM [^{3}H]-*l*-quinuclidinyl benzilate ([^{3}H]QNB) (30 Ci/mmol, New England Nuclear) ± 1 μM atropine for direct binding, and 0.07 nM [^{3}H]QNB ± appropriate concentrations of competitors for indirect binding studies. The binding was allowed to proceed for 5 hr at room temperature (or 18–20 hr at 10° when carbachol was used as a competitor), with periodic resuspension of samples by gentle inversion. Preliminary studies indicated that equilibrium had been reached in all cases, and that there were no significant differences in K_d or B_{max} between assays carried out at room temperature for 5 hr, or at 10° for 18–20 hr. In addition, temperatures lower than 37° were chosen to minimize metabolic activation of the tissue. The incubation was terminated by vacuum filtration through Whatman GF/B glass-fiber filters, followed by three washes with 4 ml KHB (4°). The filters were placed in 5 ml Liquiscint scintillation fluid and allowed to extract overnight at room temperature, followed by counting at an efficiency of 40%. Specific binding was defined as the difference between the counts in the presence and absence of 1 μM atropine, at a given concentration of [^{3}H]QNB.

Data analysis. The data from phosphoinositide turnover experiments were analyzed by computerized non-linear curve fitting to the four-parameter logistic equation of De Lean *et al.* [19], using MLAB implemented on the University of Rochester DEC-20. The fitted parameters were IC_{50} and the slope factor *b* (analogous to the Hill coefficient n_H) for a one-site model, and IC_H, IC_L (IC_{50} values for high and low affinity sites respectively) and *a* (fraction of high affinity sites) for the two-site model. In the two-site models, the slope factor *b* was constrained to unity in order to obtain an appropriate fit. The IC_{50} values for antagonists were subsequently corrected for occupancy by carbachol to yield K_i values, according to the method of Cheng and Prusoff [20].

Analysis of [^{3}H]QNB binding was performed by linear regression of a Scatchard analysis to yield

the K_d and B_{max} for [^{3}H]QNB. The inhibition of [^{3}H]QNB binding by unlabeled competitors was analyzed by computerized non-linear curve fitting to the model of Feldman [21], which takes into account the occupancy of receptor by the radiolabeled trace ligand. The fitted parameters were K_i (for a one-site model) or K_h, K_l (K_i values for high and low affinity sites respectively), B_h, and B_l (concentration of high and low affinity binding sites respectively). One-and two-site fits were compared by the use of the partial F statistic [22]. All values of EC_{50}, IC_{50}, and K_i are presented as mean (± SEM) of values obtained from best fit estimates of these parameters to individual experiments. In all cases where significant differences are claimed, the value of P is <0.05.

RESULTS

Stimulation of inositol phospholipid hydrolysis by muscarinic agonists. The hydrolysis of [^{3}H]phosphoinositides in slices of rat corpus striatum was assessed by the method of Berridge *et al.* [23] wherein the accumulation of [^{3}H]inositol phosphates ([^{3}H]IP) is measured in the presence of LiCl. Incubation of [^{3}H]inositol-labeled slices with increasing concentraions of carbachol resulted in a dose-dependent increase in the accumulation of [^{3}H]inositol phosphates (Fig. 1). The EC_{50} of carbachol for this response was 9.55 (±2.88, N = 4) × 10^{-5} M, with a maximal stimulation of 820 (±87, N = 4) % of basal [^{3}H]IP accumulation at 1 × 10^{-2} M carbachol (CCh). Computerized curve fitting of the carbachol dose–response data to a four-parameter logistic equation for interaction with a single site [15] resulted in a best fit estimate for the slope factor (mathematically analogous to the Hill coefficient used in radioligand binding studies) of −0.52 (±0.05, N = 4). However, fitting to a two-site model with the slope factors constrained to unity for both sites resulted in a significantly improved fit (P < 0.05), yielding high and low affinity EC_{50} values of 8.3 (±1.94, N = 4) × 10^{-6} M and 3.5 (±2.5, N = 4) × 10^{-3} M, respectively, with 49.5% as high affinity sites.

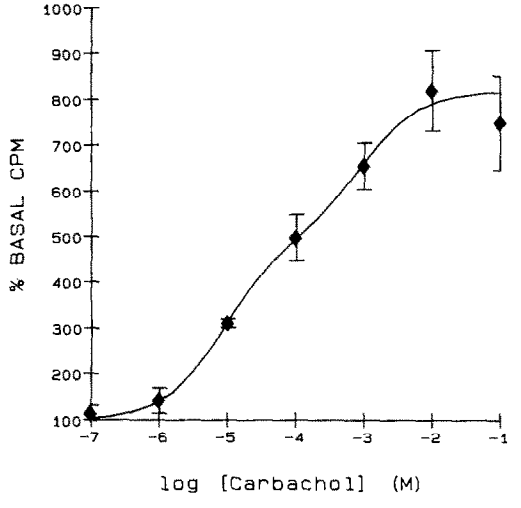

Fig. 1. Carbachol stimulation of phosphoinositide turnover. Slices (350 μm) of rat corpus striatum were labeled with [^{3}H]inositol and exposed to increasing concentrations of carbachol for 45 min at 37°. Response was measured as the accumulation of inositol phosphates (IP) in the presence of 5 mM LiCl as detailed in Methods. Basal IP production was ≈150 cpm/25 μl tissue slices, and maximal IP production was ≈200 cpm/25 μl tissue slices. Data are expressed as percent basal IP accumulation and are the mean ± SEM of four experiments performed in triplicate.

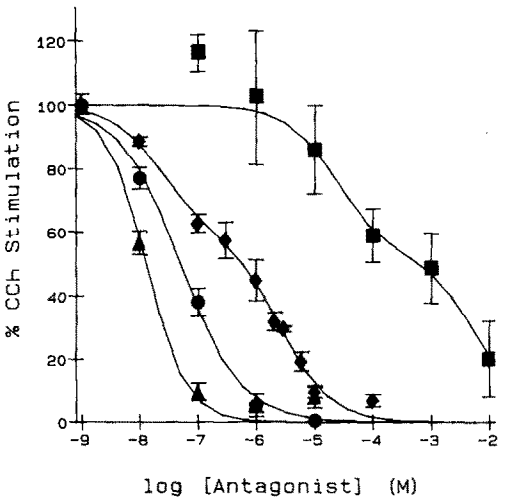

Fig. 2. Inhibition of carbachol-stimulated phosphoinositide turnover by muscarinic antagonists. Phosphoinositide turnover was stimulated by 1 × 10^{-4} M CCh in the presence of 5 mM LiCl. Various doses of atropine (▲), trihexyphenidyl (●), pirenzepine (◆), and gallamine (■) were added 10 min prior to CCh. Incubation was carried out for 45 min in the presence of carbachol. Data are presented as percent of [^{3}H]inositol phosphates accumulated in the presence of 0.1 mM CCh for 55 min and are the mean ± SEM of three experiments performed in triplicate for each antagonist.

Antagonism of carbachol-stimulated inositol phosphate production. The subtype specificity of the phosphoinositide turnover response was investigated primarily through the use of the M1-selective antagonist pirenzepine. Additional selective (gallamine) and nonselective (atropine and trihexyphenidyl) antagonists were examined in less detail (Fig. 2). Antagonist IC_{50} values were: atropine, 13.4 (±1.7) nM; trihexyphenidyl, 51.2 (±4.3) nM; pirenzepine, 0.43 (±0.057) μM; and gallamine, 0.165 (±0.078) mM.

Inhibition of carbachol-stimulated [^{3}H]IP accumulation by pirenzepine occurred in a biphasic manner (Fig. 2). The IC_{50} for pirenzepine, as determined by non-linear curve fitting assuming a single site interaction, was 4.35 (±0.57, N = 3) × 10^{-7} M, which yielded an apparent K_i of 2.12 (±0.28, N = 3) × 10^{-7} M; however, the low slope factor of 0.55 (±0.04, N = 3) and the biphasic nature of the pirenzepine inhibition curve suggested that pirenzepine was acting at more than a single site to inhibit carbachol-stimulated phosphoinositide turnover. Computerized curve fitting assuming a two-site interaction with slope factors constrained to unity improved the fit of the data to the model and gave IC_{50} values of 4.16 (±1.3, N = 3) × 10^{-8} M and 3.24 (±0.56, N = 3) × 10^{-6} M with 59 (±13, N = 3) %

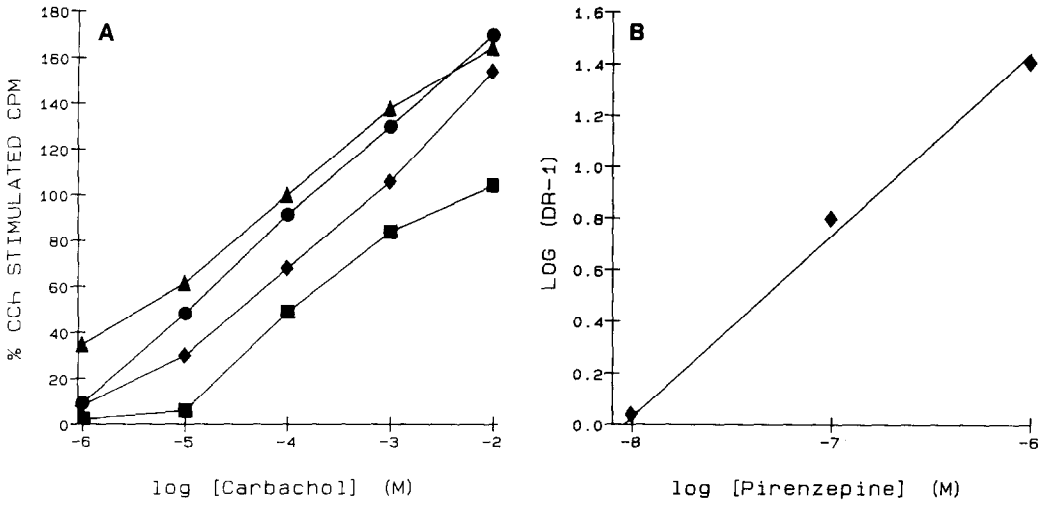

Fig. 3. Schild analysis of pirenzepine inhibition of CCh-stimulated phosphoinositide turnover. (A) Shift in the dose–response curve to CCh in the presence of increasing concentrations of pirenzepine (PZ). Slices of rat corpus striatum were labeled with [^{3}H]inositol and the response was measured by determination of the [^{3}H]IPs accumulated (as detailed in Methods) during a 45-min exposure to increasing amounts of carbachol plus 0 (▲), 10 nM (●), 100 nM (◆) or 1 μM (■) pirenzepine. Data in each curve are expressed as percent IP accumulated in the presence of 0.1 mM CCh alone and are the means of three (+PZ) or four (−PZ) experiments performed in triplicate. Standard error bars (generally <10% of mean) have been omitted for the sake of clarity. (B) Analysis of the dose–response curve shift by Schild regression. The ordinate is the log of the ratio of equiactive concentrations of CCh in the presence and absence of PZ (=DR), minus 1. The x intercept is equal to the K_i for PZ.

high affinity sites. Correction of IC_{50} values gave K_i values of 2.02 (±0.65, N = 3) × 10^{-8} M and 1.58 (±0.27, N = 3) × 10^{-6} M for high and low affinity sites respectively.

The interaction of pirenzepine with the site(s) mediating carbachol-stimulated phosphoinositide turnover was analyzed further by the method of Arunlakshana and Schild [24]. Dose–response curves for carbachol were constructed in the absence and presence of different concentrations of pirenzepine (Fig. 3A). It can be seen that pirenzepine was able to shift the carbachol dose–response curve to the right, in a dose-dependent manner. When plotted by the method of Schild (Fig. 3B) and fitted by linear regression, the x-intercept yielded a K_d for pirenzepine of 9.0 nM, and a slope of 0.7, in reasonable agreement with high affinity site by computerized curve fitting of the inhibition curve.

In contrast to the putative M1-selective antagonist pirenzepine, the putative M2-selective antagonist gallamine [16] was a very poor inhibitor of carbachol-stimulated phosphoinositide turnover. Gallamine was inhibitory only at high concentrations, the IC_{50} being 1.65 (±0.76, N = 3) × 10^{-4} M, with an apparent K_i of 8.05 × 10^{-5} M. The inhibition curve for gallamine was similar to that seen with pirenzepine in that it appeared biphasic and very shallow, with a slope factor of 0.36 (±0.09, N = 3), suggesting the possibility of a two-site interaction.

The non-selective, classical muscarinic antagonist atropine was able to inhibit completely carbachol-stimulated [^{3}H]IP accumulation in a dose-dependent manner with an IC_{50} of 1.34 (±0.17, N = 3) × 10^{-8} M. Conversion of the IC_{50} to apparent K_i using the Cheng and Prusoff correction [20] and the EC_{50} for carbachol yielded a K_i of 6.5 (±0.8, N = 3) × 10^{-9} M. Computerized curve fitting of the atropine inhibition curve resulted in a best fit slope factor of ≈1, as expected for competitive inhibition of a response by an antagonist which did not exhibit subtype selectivity.

Trihexyphenidyl (THP), which has been reported recently to exhibit a selectivity between peripheral and CNS muscarinic receptors similar to pirenzepine [25], was also able to inhibit effectively the action of carbachol on phosphoinositide turnover in a dose-dependent manner (Fig. 2). In the presence of 10^{-4} M carbachol, THP had an IC_{50} value of 5.12 (±0.43, N = 3) × 10^{-8} M and an apparent K_i of 2.5 (±0.21, N = 3) × 10^{-8} M. Computerized curve fitting indicated a best fit slope factor of 0.85 (±0.11, N = 3), which is not significantly different from 1. Thus, trihexyphenidyl does not distinguish among receptors linked to phosphoinositide turnover in the striatum.

Binding studies in intact cell aggregates. To correlate accurately the degree of receptor occupancy with the PI response mediated by muscarinic receptors in the corpus striatum, [^{3}H]QNB binding studies were performed with a tissue preparation which retained intact cells. Scatchard analysis revealed that the binding of [^{3}H]QNB to cell aggregates was saturable and of high affinity, with a K_d of 74.4 (±6.6, N = 13) pM and a B_{max} of 2.85 (±0.12, N = 13) pmol/mg protein (Fig. 4).

All competitors were able to displace completely [^{3}H]QNB bound to striatal cell aggregates (Fig. 5). The rank order of potency was the same as for the inhibition of carbachol-stimulated phosphoinositide turnover, with atropine > trihexyphenidyl > piren-

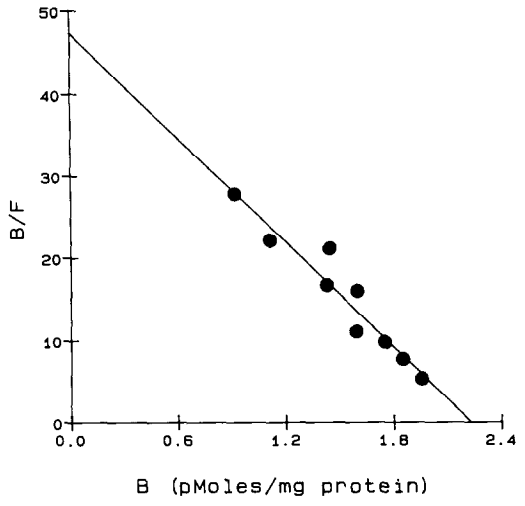

Fig. 4. Scatchard analysis of [^{3}H]QNB binding to cell aggregates from rat corpus striatum. A representative Scatchard analysis of [^{3}H]QNB binding to slices of rat corpus striatum in KHB at 21° is shown. Aliquots (50 μl) of corpus striatum cell aggregates (5–10 μg protein) were incubated in KHB with increasing concentrations of *l*-[^{3}H]QNB ± 1 μM atropine for 5 hr at 21°, in triplicate.

zepine ≫ gallamine. The inhibition curves for atropine and THP exhibited Hill slopes of 0.94 (±0.07, N = 3) and 0.87 (±0.03, N = 3), respectively, and were best described by a one-site model, with best fit estimates of the K_i equal to 0.8 (±0.04, N = 3) nM for atropine and 4.13 (±0.41, N = 3) nM for THP. The IC_{50} values were: atropine, 14 (±0.3) nM; trihexyphenidyl, 60 (±0.3) nM; pirenzepine, 2.1 (±0.2) μM; gallamine, 0.22 (±0.5) mM; and carbachol, 2.1 (±0.7) mM.

In contrast, the inhibition of [^{3}H]QNB binding to striatal cell aggregates by pirenzepine, gallamine and carbachol was complex. The Hill coefficients for these compounds were 0.78 (±0.05, N = 5), 0.66 (±0.03, N = 5), and 0.65 (±0.03, N = 5), respectively, a finding which suggests that each of these compounds is able to detect heterogeneity of the muscarinic receptors present in this preparation. Indeed, the use of a two-site model for binding resulted in significantly improved fits for each compound. Pirenzepine bound to sites with high and low affinity K_i values of 26.2 (±8.6, N = 5) nM and 251 (±58, N = 5) nM, respectively, and detected an average of 49.8 (±5.3, N = 5) % high affinity (M1) sites. Gallamine exhibited high and low affinity K_i values of 5.38 (±1.45, N = 5) μM and 247.8 (±40.1, N = 5) μM, and detected an average of 66.5 (±8.6, N = 5) % high affinity (M2) sites. In addition, in individual experiments where pirenzepine and gallamine were assayed using the same preparation, these compounds consistently detected reciprocal amounts of M1 and M2 sites. Carbachol likewise bound to two sites having high and low affinity values of 6.1 (±1.7, N = 5) μM and 270 (±67, N = 5) μM, with 51.3 (±4.2, N = 5) % high affinity sites.

DISCUSSION

It is now well established that one of the early consequences of agonist occupation of muscarinic receptors is the stimulation of phospholipase-C activity, resulting in the hydrolysis of phosphoinositides. The results of this study demonstrate that, in slices of corpus striatum, receptor occupancy by the muscarinic agonist carbachol correlates well with the stimulation of phosphoinositide turnover (Table 1). Likewise, the inhibition of carbachol-stimulated phosphoinositide turnover is generally well correlated with occupancy by muscarinic antagonists. This study also demonstrated that the M1-selective antagonist pirenzepine was much more potent than the M2-selective antagonist gallamine for both binding to receptors and inhibiting phosphoinositide turnover. However, both of the putative subtype-selective antagonists, in contrast to non-selective antagonists, inhibited phosphoinositide turnover in a complex manner.

In the mammalian CNS, carbachol has been shown to be an effective stimulator of phosphoinositide hydrolysis in tissue slices from cerebral cortex, hippocampus and neostriatum of the guinea pig [12, 26], as well as in slices from whole brain [11], cerebral cortex [9, 13], hippocampus [27], and medulla-pons [13] in the rat. The present study demonstrated that while carbachol was a potent stimulator of phosphoinositide turnover in the rat neo-striatum, it exhibited a shallow dose–response curve (slope factor = −0.53), in contrast to steep curves (slope factors ≈ 1) observed in slices of rat cerebral cortex [Monsma *et al.*, unpublished and Refs. 13 and 28] and

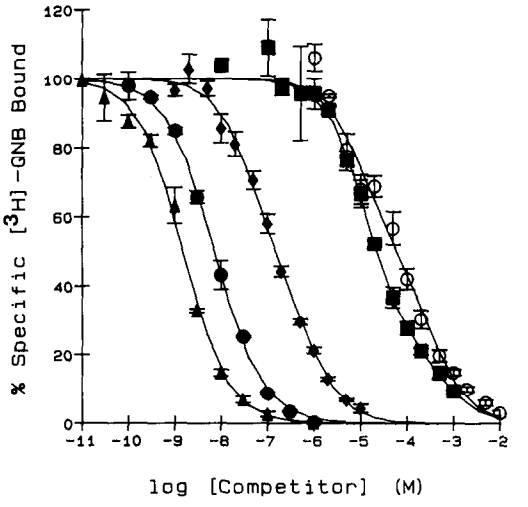

Fig. 5. Inhibition of [^{3}H]QNB binding by muscarinic agents in cell aggregates from corpus striatum. The abilities of various muscarinic agents to inhibit the specific binding of [^{3}H]QNB to cell aggregates prepared from corpus striatum were compared. Aggregates (5–10 μg protein) were incubated in KHB with 0.07 nM [^{3}H]QNB plus increasing amounts of competitor for 5 hr at 21°. Key: atropine (▲); trihexyphenidyl (●); pirenzepine (◆); gallamine (■); and carbachol (○). Data are the mean ± SEM of three separate experiments performed in triplicate and are expressed as percent specific [^{3}H]QNB binding in the absence of competitor.

Table 1. Comparison of constants derived for inhibition of carbachol-stimulated phosphoinositide turnover and inhibition of [^{3}H]QNB binding in corpus striatum

Ligand	K_h	K_l	%H	n_H
Carbachol				
PI*	8.3 ± 1.9 μM	3.5 ± 2.5 mM	49.5%	−0.52 ± 0.05
[^{3}H]QNB†	6.1 ± 1.7 μM	0.27 ± 0.67 mM	51.3 ± 4.2%	0.65 ± 0.03
Pirenzepine				
PI‡	20.2 ± 6.5 nM	1.58 ± 0.27 μM	46.4%	0.55 ± 0.03
[^{3}H]QNB	26.2 ± 8.6 nM	0.25 ± 0.058 μM	49.8 ± 5.3%	0.78 ± 0.05
Gallamine				
PI	80.5 ± 38 μM			0.36 ± 0.09
[^{3}H]QNB	5.38 ± 1.45 μM	247.8 ± 40.1 μM	66.5 ± 8.6%	0.66 ± 0.03
Atropine				
PI	6.5 ± 0.84 nM			1.25 ± 0.04
[^{3}H]QNB	0.8 ± 0.04 nM			0.94 ± 0.07
Trihexyphenidyl				
PI	24.9 ± 2.1 nM			0.85 ± 0.11
[^{3}H]QNB	4.13 ± 0.41 nM			0.87 ± 0.03

Results are best fit parameters from computerized non-linear curve fitting to one- or two-site models as described under Data Analysis. Parameters are expressed as means ± SEM of three to five individually analyzed experiments. Parameters are: K_h and K_l, high and low affinity constants respectively; %H, percent high affinity sites; and n_H, Hill coefficient for binding or slope factor for phosphoinositide turnover.

* Parameters refer to carbachol stimulation of phosphoinositide turnover.

† Parameters for all ligands vs [^{3}H]QNB refer to inhibition of 0.07 nM [^{3}H]QNB binding in striatal cell aggregates as described in Methods.

‡ Parameters for all antagonists vs carbachol-stimulated phosphoinositide turnover refer to inhibition of phosphoinositide turnover stimulated by 0.1 mM carbachol as described in Methods.

medulla-pons [13]. The dose–response relationship observed in the corpus striatum also appears to be biphasic in nature. In order to better fit this data, the four-parameter logistic equation of De Lean *et al.* [19] was expanded to include a second site. In all cases, the expanded (two-site) model provided a significantly better fit to this data. The high affinity EC_{50} value derived from this model correlates well with the high affinity site detected by carbachol inhibition of [^{3}H]QNB binding, as do the proportions of high and low affinity sites detected in both assays.

The inhibition of carbachol-stimulated phosphoinositide turnover by pirenzepine is complex and is best fit by a two-site model. The use of a two-site model is supported by Schild analysis, the results of which are consistent with the interpretation of the involvement of heterogeneous sites [29, 30]. The ability of both the M1 and M2 subtypes to be coupled to phosphoinositide turnover has been suggested previously. Based on differences in the IC_{50} for inhibition of phosphoinositide turnover by pirenzepine in different brain regions, Fisher and Bartus [12] and Lazareno *et al.* [13] have suggested that phosphoinositide turnover may be linked to more than one muscarinic receptor subtype in the CNS. In the present study, heterogeneity observed in the inhibition of carbachol-stimulated phosphoinositide turnover by selective, but not non-selective, antagonists lends support to this notion. The use of a two-site model for inhibition of phosphoinositide turnover by pirenzepine yielded a high affinity K_i value which correlates well with the value determined by [^{3}H]QNB binding and Schild analysis. Although the physiological significance of two receptor subtypes linked to the same second messenger system in the same tissue is unknown, the finding is unique and may involve receptors in two different compartments, e.g. presynaptic and postsynaptic or postsynaptic on different cells.

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