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# DIFFERENTIAL COUPLING BETWEEN MUSCARINIC RECEPTORS AND G-PROTEINS IN REGIONS OF THE RAT BRAIN

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**Abstract**—The coupling of muscarinic receptors to G-proteins in varius regions of the rat brain was assessed by measuring carbachol-stimulated, low-$K_m$ GTPase. The inhibition of carbachol-stimulated GTPase by the $M_1$-selective antagonist pirenzepine was compared to the affinity of pirenzepine for various nuclei within the regions as measured autoradiographically. The rank order of potency of carbachol for stimulating GTPase in varius brain regions was similar to that for binding to receptors in those areas. The maximal specific activity (efficacy) of carbachol-stimulated GTPase varied independently of the distribution of total receptors or receptor subtypes. The overall potency of pirenzepine for inhibiting carbachol-stimulated GTPase was not correlated with the overall affinity of pirenzepine for muscarinic receptors in the regions. Comparing results in various brain regions, the data suggest that there are differences in the efficiency of coupling between muscarinic receptors and G-proteins. For example, the pons-medulla appeared to have a small population of pirenzepine-sensitive ($M_1$ or $M_4$) receptors that were coupled very efficiently to G-proteins, whereas in the hippocampus all muscarinic receptors, most of which are pirenzepine-sensitive, appeared to be weakly coupled to G-proteins. It is suggested that variable interactions between receptors and G-proteins may be an important factor in the overall coupling between receptor occupancy and cellular responses to acetylcholine as well as other hormones and transmitters.

Recent interest has focused on the role of guanine nucleotide binding proteins (G-proteins) in coupling plasma membrane receptors to a number of intracellular processes [1]. The G-proteins that couple stimulatory ($G_s$) or inhibitory ($G_i$) receptors to adenylyl cyclase are major regulators of the synthesis of cAMP [2–4], whereas the G-protein transducin couples opsin to cGMP phosphodiesterase in retinal rod cells [5, 6]. There is also evidence that G-proteins couple receptors to the stimulation of phosphoinositude turnover [7, 8] and to cAMP phosphodiesterase in cultured cell lines [9]. In addition, data supporting the existence of G-proteins that couple receptors to $K^+$, $Ca^{2+}$ and $Na^+$ channels have been reported [10–12].

G-proteins share a common structure comprised of $\alpha$, $\beta$ and $\gamma$ subunits. The primary sequences of the $\alpha$ subunits of $G_s$, $G_i$ and transducin together with an additional protein, $G_0$ of unknown function reveal a striking homology (50–80%) among the various G-proteins [1]. The $\alpha$ subunit binds GTP in its active state. Bound GTP is cleaved to GDP by a low-$K_m$ GTPase, which is a property of the G-protein, to yield the inactive GDP by a low-$K_m$ GTPase, which is a property of the G-protein, to yield the inactive GDP bound form. Receptor-stimulated low-$K_m$ GTPase, which is a measure of receptor/G-protein interaction in brain [13], has been demonstrated for muscarinic [14] and opoid [15, 16] receptors.

There is considerable evidence for heterogeneity of muscarinic receptors in the brain based on the binding of selective ligands [17–19] and cloning of cDNAs for receptor subtypes [20–22]. In addition, muscarinic receptors appear to be coupled both to the inhibition of adenylyl cyclase [14] and the stimulation of phosphoinoside turnover [23, 24] in brain. The coupling of muscarinic receptors to G-proteins has been addressed previously by measuring the effects of guanine nucleotides on agonist binding [25]. In the present study the stimulation of low-$K_m$ GTPase by carbachol and its inhibition by the $M_1$selective antagonist pirenzepine were examined in several areas of the rat brain. The results indicate differential coupling of receptors to G-proteins among brain areas.

## MATERIALS AND METHODS

*Materials.* Male Long–Evans rats were purchased from Harlan Sprague–Dawley (Indianapolis, IN). Carbamylcholine choloride was obtained from the Aldrich Chemical Co. (Milwaukee, WI), and pirenzepine as a gift from Dr Karl Thomae, Chemisch-Pharmazeutische Fabrik, Biberach/Riss, F.R.G. [$\gamma$-$^{32}$P]-GTP was purchased from New England Nuclear (Boston, MA), and [$^3$H]-l-quinuclidinyl benzilate ([$^3$H]-QNB) from Amersham (Arlington Heights, IL). All other chemicals were obtained from the Sigma Chemical Co. (St Louis, MO).

*Tissue preparation.* Rats were killed by cervical dislocation, and their brains were rapidly removed and dissected into regions on ice by the method of Glowinski and Iversen [26]. Superior and inferior colliculi were included with brainstem (pons-medulla). Tissue from various regions was homogenized with a Brinkmann Polytron homogenizer

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(five time for 10 sec at 5-sec intervals) in 9 vol. (w/v) of a buffer solution containing 0.31 M sucrose, 0.1 mM EDTA, and 10 mM Tris–HCl (pH 7.5 at 4°).

*Preparation of brain membranes.* The crude homogenate was subjected to centrifugation for 10 min at 1000 *g*, the supernatant fraction was saved, and the pellet was resuspended in 9 vol. (w/v) of homogenization buffer and spun for another 10 min at 1000 *g*. The supernatants were combined and spun again for 30 min at 17,500 *g*. The resultant pellet was resuspended by homogenization in a Teflon–glass homogenizer in 10 vol. of TED buffer (10 mM Tris–HCl, 0.1 mM EDTA, 5 mM dithiothreitol, pH 7.5, at 4°), and washed by centrifugation at 17,500 *g* for 30 min. The final pellet was resuspended by hand homogenization with a Teflon and glass homogenizer in TED buffer. The suspension was then divided into several portions and stored at −70°.

*Determination of low-*$K_m$ *GTPase activity.* The GTPase assay was a modification [15] of methods developed by Cassel and Selinger [2] for turkey erythrocytes. The reaction was carried out in 1.5-mL microcentrifuge tubes on ice. The reaction mixture, in a total volume of 100 μL, contained 1 mM ATP, 2 μM [$\gamma^{32}$P]-GTP, 1.6 mM 5′-adenylyl-imidodiphosphate (AppNHP), 10 mM creatine phosphate, 60 units/mL creatine phosphokinase, 6 mM $MgCl_2$, 100 mM NaCl, 16 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM ethyleneglycolbis (aminoethylether) tetra-acetate (EGTA), 2 mM dithiothreitol, the appropriate concentration of [$\gamma^{32}$P]-GTP, and approximately 0.03 mg of enzyme protein. The reaction mixture (without the enzyme) was kept at 0° and then the reaction was initiated by the addition, at 20-sec intervals, of the enzyme and immediately incubated at 37°. After 10 min the reaction was terminated by the addition of 200 μL of phosphoric acid (pH 2.5), at 20-sec intervals, and transfer of the tubes to an ice bath. After 10 min, 200 μL of 2.5% (w/v) ammonium molybdate solution was added and the solution was mixed. The resultant phosphomolybdate complex was extracted into 400 μL of isobutanol, mixed for 30 sec, and then spun for 10 min at 15,600 *g* in an Eppendorf microcentrifuge. The upper (isobutanol) phase (200 μL) was transferred to plastic bags (Nalgene), and 5.0 mL of scintillation fluid was added, and the $^{32}P_i$ extracted was determined by scintillation counting. For each set of experimental conditions the contribution of high-$K_m$ GTPase was determined in the presence of 0.2 mM additional unlabeled GTP. The difference between the activity in the absence and presence of unlabeled GTP as the control represents the activity of low-$K_m$ GTP. Under the specific conditions used for measuring GTPase, the low- and high-$K_m$ values for GTP are separated by three orders of magnitude. The data indicate that subtraction of the value obtained with 0.2 mM GTP (low affinity, high-$K_m$ value) is a valid correction for the high-$K_m$ component. At this substrate level the low-$K_m$ species is far beyond saturation, and its contribution to the overall rate of hydrolysis is negligible compared with the high-$K_m$ enzyme [13].

*Autoradiographic measurements of receptor binding.* The methods for measuring inhibition of [$^3$H]-QNB binding to rat brain sections were essentially as reported previously [27, 28]. Briefly, in the experiments reported here rats were killed by cardiac perfusion with phosphate buffer, pH 7.4, followed by 0.1% formaldehyde in buffer. After removal of the brains, 12-μm sections were taken serially with a cryostat microtome at −20° and mounted on subbed (coated with gelatin and chromium potassium sulfate) microscope slides. Slides were incubated in the presence of 0.2 nM [$^3$H]-QNB and several concentrations of inhibitor. Nonspecific binding, which was measured in the presence of excess atropine, was virtually absent. After drying, slides were apposed to Ultrofilm (LKB) in the dark for 2 weeks after which the film was developed by standard photographic techniques. Images were analyzed with the Loats/Amersham RAS1000 system using optical density and tritium standards for calibration. The $IC_{50}$ and $n_H$ values were calculated from Hill plots of the inhibition data.

Under the conditions used to measure pirenzepine binding, [$^3$H]-QNB has a $K_d$ value of 0.5 nM, is completely nonselective with respect to subtypes, and displays nonspecific binding (binding to non-muscarinic receptors) of <2% of the total binding [29]. Using this method the ratio of the $IC_{50}$ values in the superior colliculus (primarily $M_2$) to that for the denate gyrus (primarily $M_1$), which is an estimate of the ratio of affinities for $M_1M_2$ receptors, is 10.0, as expected.

Table 1. Maximal activity of carbachol-stimulated low-$K_m$ GTPase in rat brain areas

| Area | Maximal activity* (nmol/mg/hr) | % Increase† |
|---|---|---|
| Cerebral cortex | 1.25 ± 0.46 | 46.4 |
| Midbrain | 0.766 ± 0.21 | 26.3 |
| Striatum | 0.687 ± 0.19 | 38.9 |
| Hippocampus | 0.561 ± 0.13 | 42.1 |
| Pons-medulla | 0.417 ± 0.09 | 41.0 |
| Cerebellum | 0.406 ± 0.16 | 18.3 |

* Maximal carbachol-stimulated low-$K_m$ GTPase activity. Values are means ± SE of 4–6 independent determinations, each performed in triplicate. The concentration of carbachol was 1 mM, which produced a maximal effect in all areas.

† Mean values of maximal carbachol-stimulated low-$K_m$ GTPase activity expressed as a percent increase over the basal, unstimulated low-$K_m$ GTPase. The basal, unstimulated low-$K_m$ GTPase values were: 0.854 (cerebral cortex), 0.606 (midbrain), 0.495 (striatum), 0.395 (hippocampus), 0.296 (pons-medulla) and 0.343 (cerebellum) nmol/mg/hr.

## RESULTS

*Stimulation of low-*$K_m$ *GTPase activity with carbachol.* Carbachol stimulated low-$K_m$ GTPase activity in all areas of the rat brain. The data in Table 1 compare the maximal stimulation of low-$K_m$ GTPase activity by carbachol in six different brain regions. The maximal specific activity of carbachol-stimulated GTPase, which varied 3-fold across different brain regions, was highest in cortex and lowest in cerebellum and medulla, with other areas having

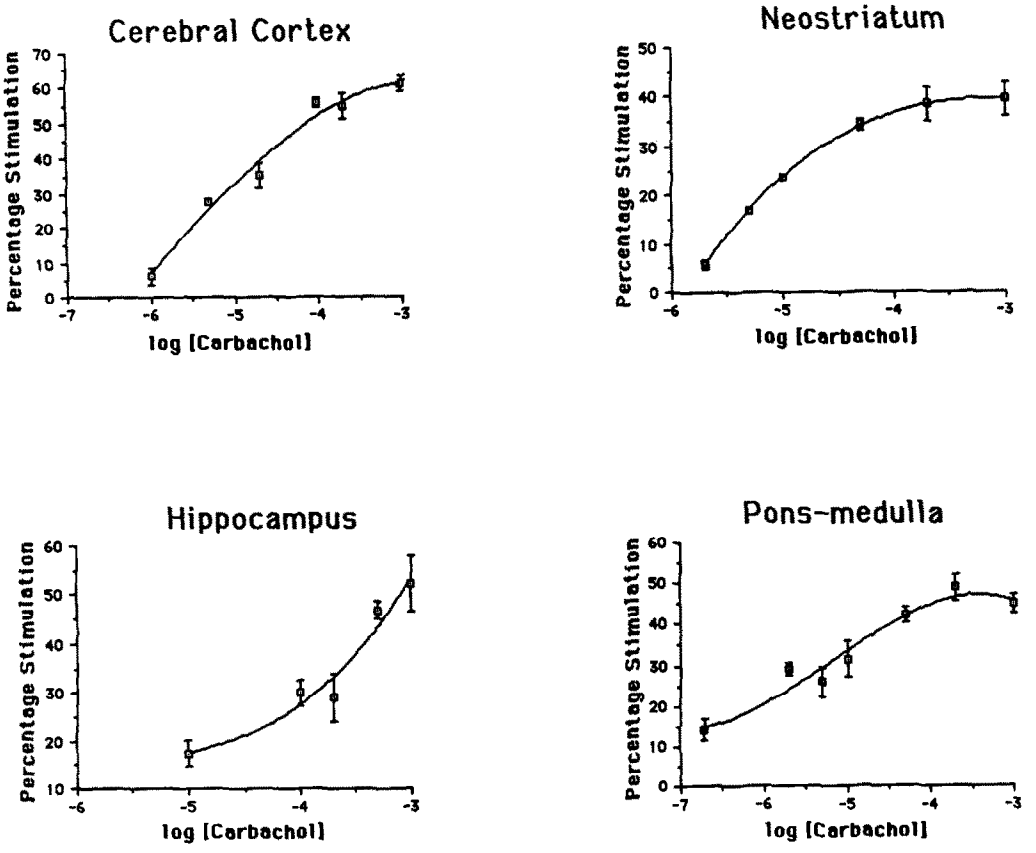


Fig. 1. Stimulation of low-$K_m$ GTPase by carbachol in regions of the rat brain. Assays were performed as described in Materials and Methods. Mean absolute values for maximal responses are shown in Table 1. Values are means ± SE, N = 3–5.

intermediate values. The regional variation of the percentage increase in carbachol-stimulated low-$K_m$ GTPase over the basal unstimulated low-$K_m$ GTPase followed the same general pattern with some exceptions (Table 1). The cerebral cortex was again highest and the cerebellum lowest, but the pons-medulla, in which carbachol-stimulated GTPase had a relatively low specific activity, displayed a high percentage increase over basal activity.

Four of the six-regions–cortex, striatum, hippocampus and pons-medulla—were selected for further study. In all areas, activity increased over a range of increasing carbachol concentrations, reached a maximal value, and then declined somewhat with higher concentrations of carbachol (Fig. 1). The $EC_{50}$ values varied greatly among the different regions giving the order of potency pons-medulla > cortex > striatum ≫ hippocampus (Table 2). Hill slopes for the stimulation of low-$K_m$ GTPase by carbachol were significantly less than 1 in all areas except cortex (Table 2), suggesting that more than one muscarinic receptor/G-protein interaction is present in most areas of brain.

*Inhibition of carbachol-stimulated GTPase by pirenzepine.* Pirenzepine, which is an $M_1/M_4$-selective (but not specific) muscarinic receptor antagonist, inhibited carbachol-stimulated GTPase activity in the four brain areas in a dose-dependent manner

Table 2. $EC_{50}$ Values for the stimulation of low-$K_m$ GTPase by carbachol

| Area | $EC_{50}$ ($\mu$M) | $n_H$ |
|---|---|---|
| Cerebral cortex | 7.73 ± 1.90 | 0.918 ± 0.107 |
| Hippocampus | 199 ± 68 | 0.629 ± 0.224 |
| Pons-medulla | 4.74 ± 0.46 | 0.462 ± 0.186 |
| Striatum | 13.9 ± 1.8 | 0.505 ± 0.032 |

Values are means ± SE of 3–5 independent determinations, each performed in triplicate.

(Fig. 2). The $IC_{50}$ values varied approximately 1000-fold to yield the order of potency pons-medulla ≫ cortex > striatum > hippocampus (see Table 3). Hill coefficients were less than 1 in all areas except for the pons-medulla.

*Binding of pirenzepine to muscarinic receptors.* The relative potency of pirenzepine for inhibiting carbachol-stimulated GTPase was compared to the affinity of pirenzepine for muscarinic receptors in several brain areas. The $IC_{50}$ values for inhibition of [$^3$H]-QNB binding were measured in brain sections by quantitative autoradiography in various nuclei within the four regions in which GTPase was determined (Table 4). The $IC_{50}$ values of pirenzepine for

Table 3. Inhibition of carbachol-stimulated GTPase by pirenzepine

| Area | $IC_{50}$ ($\mu$M) | $n_H$ |
|---|---|---|
| Cerebral cortex | 0.82 ± 0.02 | 0.521 ± 0.048 |
| Hippocampus | 29.0 ± 4.1 | 0.520 ± 0.129 |
| Pons-medulla | 0.027 ± 0.006 | 1.58 ± 0.08 |
| Striatum | 3.10 ± 0.03 | 0.696 ± 0.165 |

Values are means ± SE of 3–4 independent determinations, each performed in triplicate. The concentration of carbachol was 1 mM.

muscarinic receptors varied approximately 10-fold overall to give the order of affinity hippocampus > striatum > cortex > pons-medulla. The data also indicated areas within the pons-medulla that have a relatively high affinity for pirenzepine.

## DISCUSSION

Carbachol-stimulated GTPase represents the interaction between muscarinic receptors and G-proteins. Variables comparing different brain areas include affinity of carbachol for the receptors (dependent on the relative distribution of receptor subtypes), efficiency of coupling between receptors and G-proteins, receptor density, and distribution of G-proteins in that area. Since the other parameters are generally known, carbachol-stimulated GTPase can serve as a measure of the efficiency of receptor/G-protein interaction.

The rank order of potency of carbachol for stimulating low-$K_m$ GTPase in various brain regions generally corresponded to the rank order of affinity of carbachol for muscarinic receptors in the regions—highest in pons-medulla, lowest in hippocampus and intermediate in cortex and striatum. These findings are consistent with the notion that carbachol is an $M_2$-selective agonist, having a greater potency for stimulating GTPase in areas (e.g. pons-medulla) rich in $M_2$ receptors. In addition, low $n_H$ values of carbachol-stimulated GTPase are typical of those found in binding studies and suggest interaction with multiple receptor sites. Preliminary data show no evidence for desensitization of carbachol-stimulated GTPase in the brain (Ghodsi-Hovsepian *et al.*, unpublished results).

On the other hand, maximal carbachol-stimulated low-$K_m$ GTPase activity, expressed as carbachol-stimulated specific activity or percentage increase over basal, unstimulated, low-$K_m$ GTPase, did not

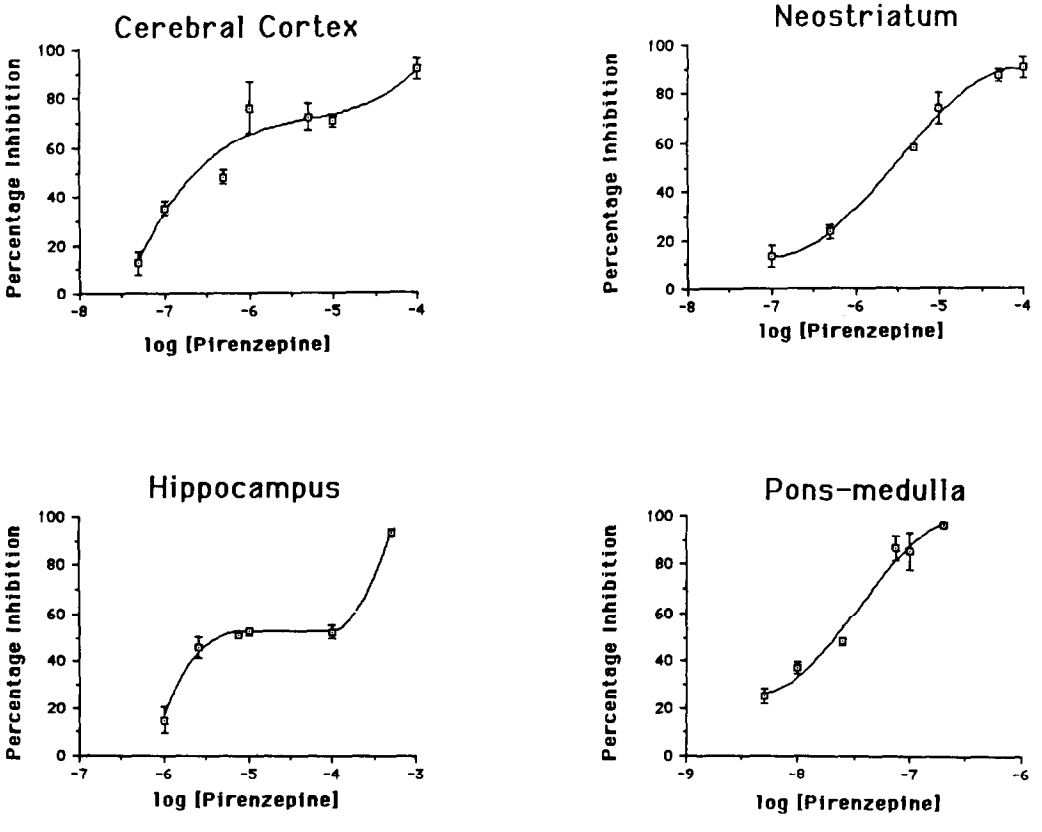


Fig. 2. Inhibition of carbachol-stimulated GTPase by pirenzepine in regions of the rat brain. Assays were performed as described in Materials and Methods. The concentration of carbachol was 1 mM. Values are means ± SE, N = 3–4. The values of carbachol-stimulated GTPase in the absence of pirenzepine were the same as the maximal activity values shown in Table 1.

Table 4. Binding of pirenzepine to several brain regions

| Area | $IC_{50}$ ($\mu$M) | $n_H$ |
|---|---|---|
| Cerebral cortex | | |
| Layers I–III | 0.44 ± 0.06 | 0.76 ± 0.06 |
| Layers IV and V | 0.48 ± 0.05 | 0.76 ± 0.04 |
| Layer VI | 0.52 ± 0.07 | 0.85 ± 0.07 |
| Hippocampus | | |
| Dentate gyrus | | |
| Dorsal | 0.19 ± 0.00 | 0.95 ± 0.09 |
| Ventral | 0.21 ± 0.04 | 0.98 ± 0.14 |
| CA1 | 0.29 ± 0.02 | 0.93 ± 0.06 |
| CA3 | 0.27 ± 0.02 | 0.83 ± 0.05 |
| CA4 | 0.29 ± 0.05 | 0.90 ± 0.04 |
| Striatum | | |
| Dorsomedial | 0.31 ± 0.05 | 0.75 ± 0.04 |
| Ventromedial | 0.79 ± 0.11 | 0.85 ± 0.05 |
| Pons-medulla | | |
| Superior colliculus | 2.0 ± 0.26 | 1.29 ± 0.27 |
| Substantia nigra | 0.27 ± 0.06 | 0.55 ± 0.06 |
| Periaqueducatal grey | 1.0 ± 0.08 | 1.05 ± 0.16 |
| Dorsal raphe nucleus | 1.1 ± 0.27 | 0.91 ± 0.00 |
| Inferior colliculus | 0.57 ± 0.06 | 0.87 ± 0.16 |
| Pontine nuclei | 1.2 ± 0.25 | 1.32 ± 0.24 |

Values are means ± SE of triplicate determinations for at least six concentrations of pirenzepine from three animals.

correspond to the distribution of total muscarinic receptors (or $M_1$–$M_4$ receptor subtypes) determined from binding studies and *in situ* hybridization studies. This finding is indicative of overall differences in coupling efficiencies between G-proteins and receptors comparing various brain regions. These data may be related to the finding that the cerebellum, which contains one of the lowest densities of muscarinic receptors in the brain, shows the greatest response to carbachol-stimulated prostaglandin formation [30]. It is also possible that there are spare receptors for the GTPase response. This could vary among different brain regions.

The binding of pirenzepine to muscarinic receptors was compared with the ability of pirenzepine to inhibit low-$K_m$ GTPase activity since both measure the potential interaction of pirenzepine with all muscarinic receptors. The inhibition of carbachol-stimulated GTPase activity by the $M_1$/$M_4$-selective antagonist pirenzepine in a particular brain region compared with the binding of pirenzepine to nuclei within the region is a measure of the relative involvement of $M_1$/$M_4$ receptors on the one hand and $M_2$/$M_3$ receptors on the other hand in the muscarinic receptor-stimulated GTPase response in that region. The lack of correlation between the potency of pirenzepine for binding to muscarinic receptors and potency for inhibiting carbachol-stimulated GTPase suggests that $M_1$/$M_4$ receptors are coupled to G-proteins with different efficiencies in different areas. For example, the 1000-fold greater potency of pirenzepine in the pons-medulla compared with the hippocampus was unexpected from a 10-fold difference in binding affinities, especially in view of the finding that pirenzepine had a higher affinity for the hippocampal receptors. The inhibition curves for hippocampus and cortex appear to be biphasic. Estimating the lower $IC_{50}$ value in the hippocampus as 2 $\mu$M, a 100-fold difference between that value and the $IC_{50}$ value for the pons-medulla remains.

It is possible that a relatively small population of $M_1$ or $M_4$ receptors in pons-medulla may be very efficiently coupled to G-proteins, whereas the relatively large numbers in the hippocampus are less efficiently coupled. The binding data revealed areas, for example the inferior colliculus, that have a relatively high affinity for pirenzepine within the pons-medulla (brainstem). Further work may identify specific nucleii within the pons-medulla having relatively high densities of $M_1$ or $M_4$ receptors that are very efficiently coupled to G-proteins. Recent studies using muscarinic receptor subtypes expressed in cell culture have suggested that $M_1$ and $M_3$ receptors are coupled to phosphoinositide metabolism whereas $M_2$ and $M_4$ receptors are coupled to the inhibition of adenylyl cyclase [31]. The data from pons-medulla and hippocampus suggest that muscarinic receptors are coupled efficiently to the inhibition of adenylyl cyclase through both $M_2$ and $M_4$ receptors and less efficiently to guanine nucleotide binding proteins mediating phosphoinositide metabolism by activation of $M_1$ and/or $M_3$ receptors.

The reason for differences in coupling efficiencies between muscarinic receptors and G-proteins in different brain regions is presently unclear. The expression of at least four different muscarinic receptors in the rat brain [20, 22, 32] together with the presence of at least four different G-proteins which have different regional distributions [33] and perhaps different intrinsic GTPase activities, however, does allow for a substantial number of different combinations of muscarinic receptors and G-proteins. Regardless of the molecular explanation for differences in coupling efficiencies between muscarinic receptors and G-proteins in different brain areas,

the differences are revealed by measuring receptor-mediated GTPase. On the other hand, guanine nucleotide effects on muscarinic receptor binding in brain have not detected these differences. The finding that three of the four receptors expressed in the rat brain have a relatively high affinity for pirenzepine (at least when expressed in cultured cell lines) [20] indicates that new ligands with greater selectivity will be required to establish the links among specific receptor subtypes, G-proteins and second messenger systems in a particular brain area. In addition, differences in coupling between receptors and G-proteins may contribute to observed heterogeneity in some muscarinic responses within a single brain region [34] as well as discrepancies between receptor occupancy and response [29].

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