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Differences in Ligand Binding and Phosphoinositide Turnover Between M_1 Muscarinic Receptor Gene Transfected Cells and Mouse and Rat Brain Membranes

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WANG, D. X., N. LERNER-MARMAROSH, S.-S. SHEU, V. SHARMA, M.-J. JOU AND L. G. ABOOD. *Differences in ligand binding and phosphoinositide turnover between M_1 muscarinic receptor gene transfected cells and mouse and rat brain membranes.* PHARMACOL BIOCHEM BEHAV 49(2) 405–411, 1994.—The present study describes some unexpected receptor mediated effects of *N*-methylcarbamylcholine on mouse M_1 muscarinic receptor gene transfected cell line (M_1Y_1) that were not evident from biochemical studies with mouse and rat brain tissue where *N*-methylcarbamylcholine exhibited only nicotinic properties. Although *N*-methylcarbamylcholine was devoid of muscarinic properties in mouse and rat brain preparations, as determined by phosphoinositide turnover and inhbiiton of [^{3}H]QNB binding, it exhibited significant muscarinic characteristics in the transfected M_1Y_1 cell line. At a concentration of 10^{-6} M or greater, *N*-methylcarbamycholine caused a transient increase in intracellular Ca^{2+} of 50 s duration that was reversible by atropine or pirezepine. The Ca^{2+} transient was not elicited by other nicotinic agents such as nicotine and *N,N*-dimethylcarbamylcholine, a close analogue of *N*-methylcarbamylcholine, with comparable affinity for nicotinic receptors and devoid of muscarinic activity. *N*-Methylcarbamylcholine also stimulated phosphoinositide turnover in M_1Y_1 cells with an estimated EC_{50} value 10 times greater than that of carbachol, and the effect was blocked by atropine. Both carbachol and *N*-methylcarbamylcholine inhibited [^{3}H]QNB binding in a concentration-dependent manner; however, the IC_{50} for carbachol was over two orders of magnitude greater than that observed in mouse and rat brain membranes. In considering possible explanations for the differential characteristics of *N*-methylcarbamylcholine in mouse and rat brain as compared to the transfected M_1Y_1 cells, it was concluded that the difference may be attributable to differences in the receptor-transduction coupling effeciency and the microenvironment of the muscarinic receptors.

Muscarinic receptors *N*-Methylcarbamylcholine phosphoinositide Calcium transients Transfected M_1 gene

CURRENT understanding of nicotinic and muscarinic cholinergic receptors is based on their molecular structure, receptor mediated physiological response, and pharmacological characteristics. Nicotinic acetylcholine receptors are ion channel-linked receptors, selectively activated by nicotine and controlling cation permeability. Muscarinic acetylcholine receptors (MAChRs) are G protein-mediated receptors linked to various secondary messenger systems. Knowledge of the functional diversity of the various subtypes of nicotinic and muscarinic cholinergic receptors has depended largely on the discovery of selective agonists and antagonists. The study of biochemical and functional characteristics of cholinergic receptors has greatly accelerated with the development of receptor DNA transfected cell lines. Among the many advantages of trans-

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fected cell lines is that they insure a homogeneous population of cells for examining the biochemical and functional properties of a single receptor subtype.

Recently it was noted that the addition of an alkyl substituent on the carbamyl N of carbamylcholine abolished muscarinic activity and significantly enhanced nicotinic potency (1). Included among this class of agents were *N*-methylcarbamylcholine (MCC) and ethylcarbamylcholine, both having affinities for nicotinic cholinergic receptors approaching that of nicotine. The present study describes some unexpected receptor-mediated effects of MCC on a mouse M_1 muscarinic receptor gene transfected cell line (M_1Y_1) that were not evident from biochemical studies with mouse and rat brain tissue.

METHOD

Materials

Mouse M_1 MAChR genomic DNA cloned in an expression vector containing mouse metallothionein-1 promoter (M_1/ZEM228) was a gift from Neil M. Nathanson (13).

The mouse adrenal cell line Y_1 was purchased from American Tissue Culture Corporation. [^{3}H]-3-Quinuclidinyl benzilate ([^{3}H]QNB) was purchased from New England Nuclear. *Myo*-[2-^{3}H]Inositol was from Amersham. Cell culture reagents including F-10 medium, horse serum, fetal bovine serum, and gentamicin were from Gibco.

Cell Culture

The cells were cultured in F-10 medium containing 15% horse serum and 2.5% fetal bovine serum, 50 μg/ml gentamicin at 37°C and 5% CO_2. Cells were subcultured every 5 days. M_1/ZEM228 DNA was subsequently transformed into *E. coli* JM-109 (from Promega Corp.), cloned, and amplified in LB broth media containing chloramphenicol. The DNA was harvested using conventional methods and purified by the cesium chloride technique (12). The M_1/ZEM228 gene was transfected into Y_1 cells using the Ca phosphate method (14) with slight modifications. Briefly, 5×10^6 cells were plated in 100 mm plates 24 h before transfection and the medium was changed 4 h prior to transfection. The DNA/Ca phosphate precipitate was prepared by dropwise addition of 20 μg of vector plasmid DNA dissolved in 2 ml of 0.1265 M $CaCl_2$ into 2 ml of Hepes-phosphate buffer containing 1.4 mM sodium phosphate, 50 mM Hepes, and 280 mM NaCl, pH 7.05–7.10. After the mixture was kept at room temperatue for 20 min, 2 ml of the DNA/Ca phosphate precipitate was used to transfect cells in each 100 mm dish prepared as described above. After 4 h of incubation in 37°C, the cells were rinsed with serum-free F-10 medium and cell culture medium was replaced. Stable transfected cells were selected 2 days later by using a medium containing 500 μg/ml gentamicin (as biological activity). After 2 weeks, the surviving cells were trypsinized and subcultured in the gentamicin containing medium at a very low density so that isolated colonies developed from single cells could be recognized and selected. Individual cell strains developed from single cell colonies were screened for [^{3}H]QNB binding study, and several cell strains that expressed the highest level of receptor were chosen for later experiments.

[^{3}H]QNB Binding

Cells were allowed to grow in 150 mm dishes to 80% confluence, and after exposure to the same medium containing 110 μM zinc sulfate for 24 h, the dishes were washed twice with the phosphate-buffered saline. The cells were then transferred to a centrifuge tube and centrifuged at 1000 rpm for 5 min. After gently homogenizing with a glass homogenizer in 50 mM Tris buffer, pH 7.5, the contents were recentrifuged at 12,000 rpm for 15 min and the pellet resuspended in the Tris buffer. [^{3}H]QNB binding was performed in a total volume of 2 ml containing approximately 30 μg of membrane protein and various concentrations of [^{3}H]QNB (45 Ci/mmol) with and without a 100-fold greater concentration of unlabled QNB. When MCC or carbachol inhibition was determined, the membranes were preincubated with either agent for 30 min prior to the addition of [^{3}H]QNB. After 2 h of incubation at room temperature, the contents were filtered in vacuo through Whatman GB/F glass filters, washed with 8 ml of ice-cold 50 mM Tris buffer pH 7.5, and measured for radioactivity by liquid scintillation counting.

Brain membranes were prepared from Swiss colony adult male mice using procedures for rat brain described elsewhere (1).

Intracellular Ca^{2+} Transients

The cells were seeded on sterile coverslips (25 mm circule #1, VWR, San Francisco, CA) and allowed to grow to 30% confluence. Approximately 24 h before use, the tissue culture medium was replaced by the one containing 110 μM zinc sulfate. Cellular Ca^{2+} measurements were were performed as described elsewhere (15). Briefly, the cells were washed once with the Hepes buffer and loaded with 1 μM fura-2 AM for 1 h at room temperature. The coverslip was then fixed in a tissue chamber (1.0 ml in volume) and mounted on the stage of a Nikon inverted microscope equipped for epifluorescence. At the beginning of each experiment a camera (SIT Camera, Dage MTI 65, Michigan City, IN) gain setting was chosen for the experiment. This setting was below the level at which auto fluorescence from unloaded cells will be detected and precluded the possibility of signal saturation. The cells were excited sequentially at 380 and 340 nm from a 75 watt Xenon lamp and the emitted fluorescent images were acquired at 510 nm with a dichroic mirror (Omega Optical, Brattleboro, VT). The acquired images were digitized by an image processor (FG-100-1024, Imaging Technology, Woburn, MA), housed in a microcomputer, at a pixel resolution of 512 horizontal × 512 vertical. The fluorescent ratio image was then obtained by dividing the 340 nm image by 380 nm image on a pixel-by-pixel basis. Quantification of [Ca^{2+}] was obtained from the equation

$$[Ca^{2+}] = K_d \times \{(R - R_{min})/(R_{max} - R)\} \times Sf2/Sb2$$

where R is the measured cellular ratio and R_{min} and R_{max} are the ratios obtained in $[Ca^{2+}]_i$ free and saturating [Ca^{2+}]. Sf2 in the 380 nm excitation signal in the absence of Ca^{2+}, and Sb2 is the 380 nm excitation signal at saturating [Ca^{2+}] in high K^+ Hepes buffer which contained 140 mM KCl, 10 mM NaCl, 1 mM K_2 EGTA, 1 mM $MgCl_2$, 10 mM Hepes, and 3 μM fura 2 pentapotassium salt (8).

Phosphoinositide Turnover

Phosphoinositide (PI) turnover was performed by the method of Shapiro et al. (13) with slight modifications. Cells were grown to 70% confluence in 12 well cell culture plate, followed by 24 h incubation in cell-culturing medium containing 110 μM zinc sulfate and [^{3}H]-inositol at 2 μCi/ml. They were then rinsed twice with saline solution containing 118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgSO_2$, 1.2 mM

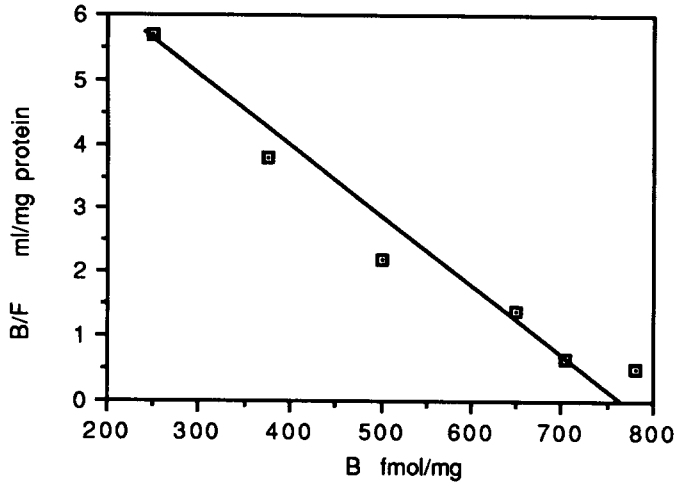

FIG. 1. Scatchard plot of [^{3}H]QNB binding in M_1Y_1 cells. The plot is representative of three separate experiments each in triplicate.

KH_2PO_4, 10 mM glucose, 0.5 mM EDTA, and 20 mM Hepes, pH7.4, followed by incubation in physiological saline solution containing, in addition, 10 mM LiCl for 30 min at 37°C. Varying concentrations of cholinergic agents were then added and the cells were incubated for an additional 15 min. The reaction was terminated by removing the supernatant and adding 0.6 ml of ice cold methanol into the wells. After the cells were scraped off from the wells and transferred to centrifuge tubes, 0.3 ml each of chloroform and water were added, and the tubes vortexed vigorously to break up the cells. After the addition of another 0.3 ml each of chloroform and water, the tubes were again vortexed and centrifuged at 1500 × *g* for 15 min. [^{3}H]-Inositol phosphates were then determined in the supernatant by fractionation on a Dowex 1-X8 column (13).

PI turnover in mouse cerebral brain slices was performed as described elsewhere (3).

RESULTS

[^{3}H]QNB Binding

After transfection and gentamicin selection, several strains of cells (M_1Y_1) exhibited a significant increase of [^{3}H]QNB binding compared to wild type Y_1 cells. A Scatchard analysis of [^{3}H]QNB binding performed on the strain with the highest binding capacity revealed a linear plot with a K_d value of 100 pM and B_{max} 800 fmol/mg (Fig. 1). [^{3}H]QNB binding was completely inhibited by 1×10^{-6} M pirenzepine, a M_1 selective ligand (data not shown).

The IC_{50} values for carbachol and MCC inhibition of [^{3}H]QNB binding to mouse brain membranes were 8×10^{-7} M and 1×10^{-4} M, respectively (data not shown).

Carbachol and MCC-Stimulated Ca^{2+} Transients

Ca^{2+} mobilization in M_1 transfected Y_1 cells in response to different cholinergic agents was determined with a fluorescence digital microscopic imaging system. A concentration of 1×10^{-6} M or greater MCC caused a transient increase in intracellular Ca^{2+} of 50-s duration, the magnitude of the second response after washing being identical to the first one (Fig. 2). The form of the response was similar to that of

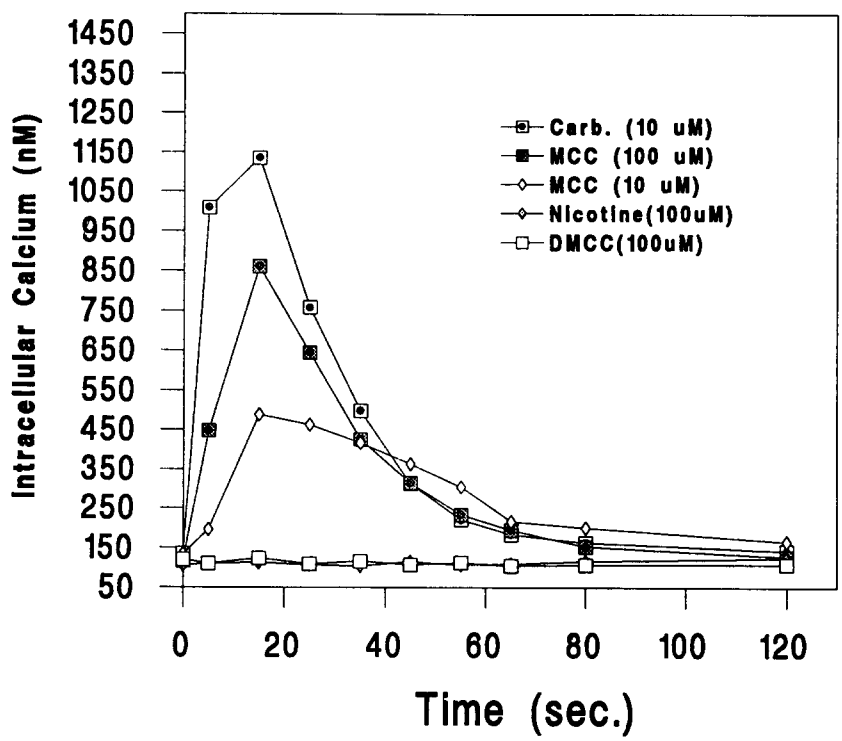

FIG. 2. Intracellular Ca^{2+} transients of M_1Y_1 cells in response to carbachol, MCC, nicotine and *N,N*-dimethylcarbamylchline (DMCC). The data is based on four experiments agreeing within 6%.

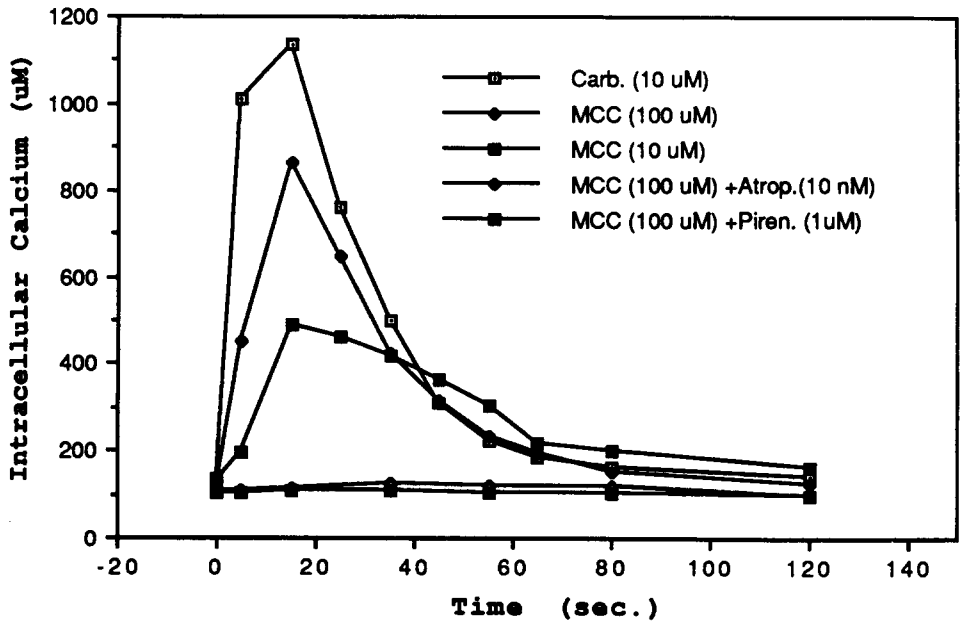

FIG. 3. Antagonism of intracellular MCC-induced Ca^{2+} transients by atropine and pirenzepine in M_1Y_1 cells. The data is based on three separate experiments agreeing within 7%. Atrop = atropine; pirenz. = pirenzepine. Data for the effect of carbachol and various concentrations of MCC alone taken fron Fig. 2.

carbachol, except that the potency of of carbachol was 5 times higher than that of MCC. The Ca^{2+} transient was not elicited by other nicotinic agents such as nicotine, *N*-ethyylcarbamylcholine, and *N,N*-dimethylcarbamylcholine at a concentration of 1×10^{-4} M. The increase in the Ca^{2+} transient by MCC was completely blocked by 1×10^{-8} M atropine or 1×10^{-6} M pirenzepine (Fig. 3).

Carbachol and MCC-Stimulated Phosphoinositide Turnover

Both carbachol and MCC stimulated cellular PI turnover in M_1Y_1 cells: the estimated EC_{50} value for MCC being approximately 10 times greater than that of carbachol (Fig. 4). The stimulated PI turnover of both agents was blocked atropine (Fig. 5). The combined effect of carbachol and MCC at maximally effective concentrations on PI turnover was greater than that of each alone but less than the sum (Fig. 6); whereas at lower concentration, their combined response level was additive. *N*-Ethylcarbamylcholine and *N,N*-dimethylcarbamylcholine at 1×10^{-4} M were inactive on PI turnover (data not shown).

MCC at concentrations as high as 1×10^{-3} M was without effect of PI turnover of mouse cortical brain slices; while the EC_{50} for carbachol stimulated PI turnover was 7×10^{-5} M (data not shown).

Effect of MCC on [^{3}H]MCC Binding

In order to determine whether MCC interacted with M_1 receptors, [^{3}H]MCC binding studies were performed with essentially negative results with both membrane and intact cells. On the other hand, MCC was found to inhibit [^{3}H]QNB binding in a concentration dependent mode (Fig. 7).

DISCUSSION

In the course of screening various cholinergic agents for their effect on intracellular Ca^{2+} transient, it was noted that MCC caused a significant physiological response in M_1 muscarinic receptor gene transfected cells. The finding was unexpected since MCC was believed to be devoid of muscarinic activity (3). Both receptor binding and pharmacological studies revealed MCC to be a potent nicotinic agonist, and [^{3}H]MCC has become a useful ligand for neuronal nicotinic receptors (1,2). Although the potency of MCC on Ca^{2+} transients was less than 1/10 that of carbachol, the finding was of interest because of the MCC's presumed lack of muscarinic activity. One explanation for the finding was that MCC may be acting directly on receptor mediated ion channels. Because neither MCC nor nicotine produced a Ca^{2+} transient in wild type Y_1 cells, this explanation is not likely. The fact that MCC increased PI turnover in transfected cells indicated that the effect was via a G protein-mediated signal transduction pathway. The question remained as to whether the effect of MCC on M_1Y_1 cells was mediated directly via the transfected M_1 receptor. There were a number of similarities in the action of carbachol and MCC on Ca^{2+} transients and PI turnover. The time course of Ca^{2+} transient was identical after carbachol and MCC stimulation and the stimulation by both agents was concentration dependent. The combined effect of both was additive at lower concentration but not at higher concentration, a finding that indicates that their effects are complementarily saturable. Insofar as the effect was blocked by atropine and pirenzepine, it appears to be receptor mediated. The ratio of IC_{50} values for MCC and carbachol inhibition of [^{3}H]QNB binding in M_1Y_1 cells was about 10 (1×10^{-3} M vs. 1×10^{-4}

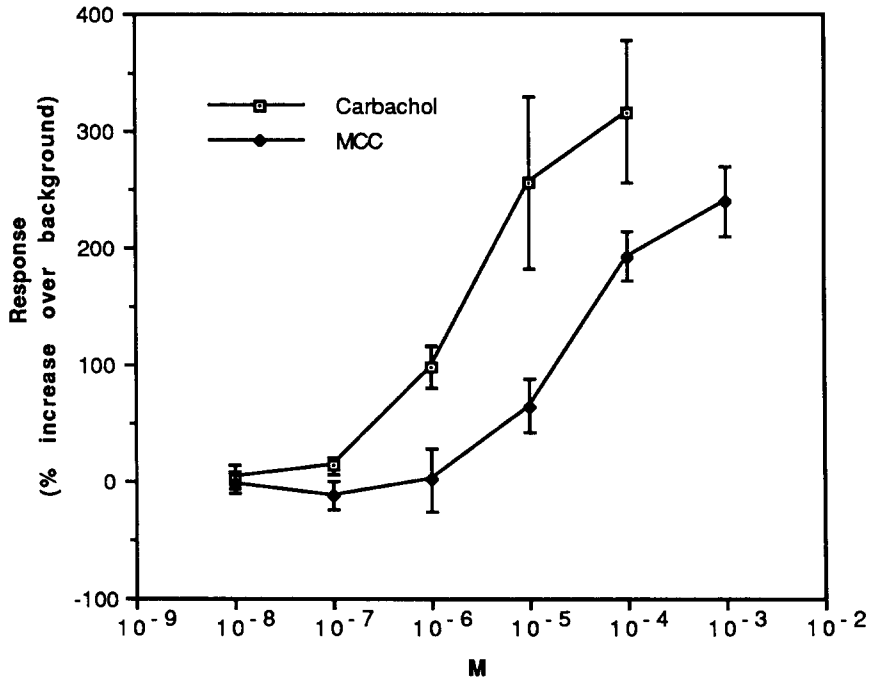

FIG. 4. Concentration response curve of PI turnover stimulated by carbachol and MCC in M_1Y_1 cells. The response was defined as the percentage of increased [^{3}H]-phosphoinositide (PI) formation over background.

M), which is in agreement with the ratio of apparent EC_{50} values for MCC (5×10^{-4} M) and carbachol (5×10^{-5} M) stimulated PI turnover. Another difference between the brain and M_1Y_1 cells was that the IC_{50} for carbachol inhibition of [^{3}H]QNB binding of both mouse and rat brain preparations was two orders of magnitude less than the value for M_1Y_1 cells; the values being comparable for mouse and rat brain (1). Because of the relatively low affinity of MCC for the M_1 transfected receptor, attempts to determine [^{3}H]MCC binding to the M_1 transfcted cells were unsuccessful. Evidently, as a result of the low affinity of MCC, the receptor bound [^{3}H]MCC dissociated from the receptors. during the procedure of removing free ligand from the reaction. On the basis of the foregoing evidence it could be inferred that MCC stimulated M_1 transfected Y_1 cells via M_1 receptors, and probably acted on the same affinity recognition site as prototypic muscarinic ligands; however, several questions still remain.

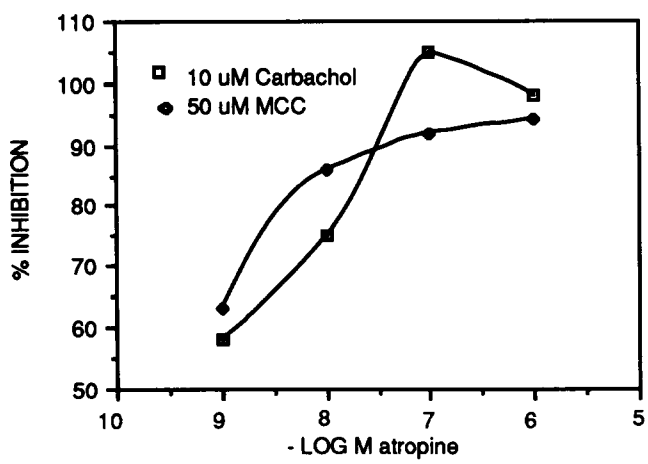

FIG. 5. Inhibition by atropine of carbachol and MCC simulated PI turnover in M_1Y_1 cells. The results are an average of two separate experiments agreeing within 7 %.

Firstly, MCC fails to stimulate PI turnover in mouse or rat brain slices (3). Because the density of brain muscarinic receptors (B_{max} = 3 pmol/mg) is comparable to that of the transfected M_1Y_1 cells (B_{max} = 0.8 pmol/mg), the difference cannot be attributed to receptor density. Another explanation may be that the coupling effeciency between the M_1 receptor and transduction mechanisms is greater in the transfected cells. It has been reported that M_1 coupling effeciency in brain is poor as compared to other tissues (6,7) and that muscarinic agonists that are relatively inactive in brain are effective in transfected cells (9) The fact that the IC_{50} value for carbachol inhibition of [^{3}H]QNB binding of mouse as well as rat brain preparations (5×10^{-7} M (3) was two orders of magnitude less than the value for M_1Y_1 cells is consistent with the notion of a lower transduction efficiency in brain as compared to the M_1Y_1 cells. Conceivably, the microenviroment in the vicinity of the brain and transfected receptors differs in such a way as to affect ligand selectivity or the interaction between the recognition site, G protein, and phospholipase C. It has been reported that antagonist binding properties may vary accord-

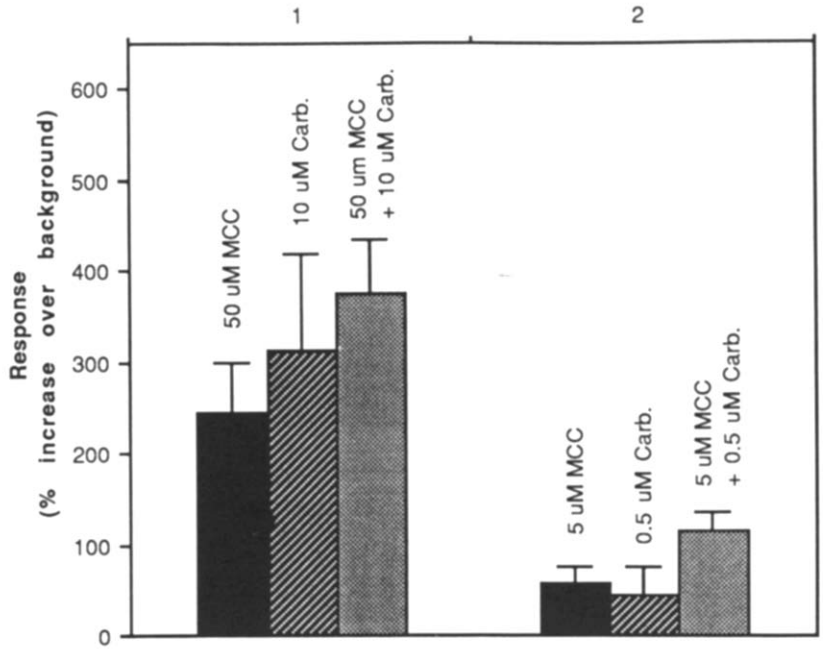

FIG. 6. The combined effect of varying concentrations of carbachol and MCC on PI turnover in M_1Y_1 cells. The response was expressed as the percentage of the increased PI formation over the background.

ing to the cellular environment in which the receptor is placed (5). *N*-Ethylcarbamylcholine and *N,N*-dimethycarbamylcholine, which differ slightly from MCC in the carbamyl *N*-alkyl group and have similar K_i values on [^{3}H]QNB and [^{3}H]MCC binding (11), are both inactive on PI turnover and Ca transients in transfected M_1Y_1 cells. Conceivably, steric hindrance resulting from the more bulky *N*-alkyl substituents prevents the ligand-mediated PI response.

Another possible explanation for the differences observed in the effects of MCC on mouse and rat brain preparations

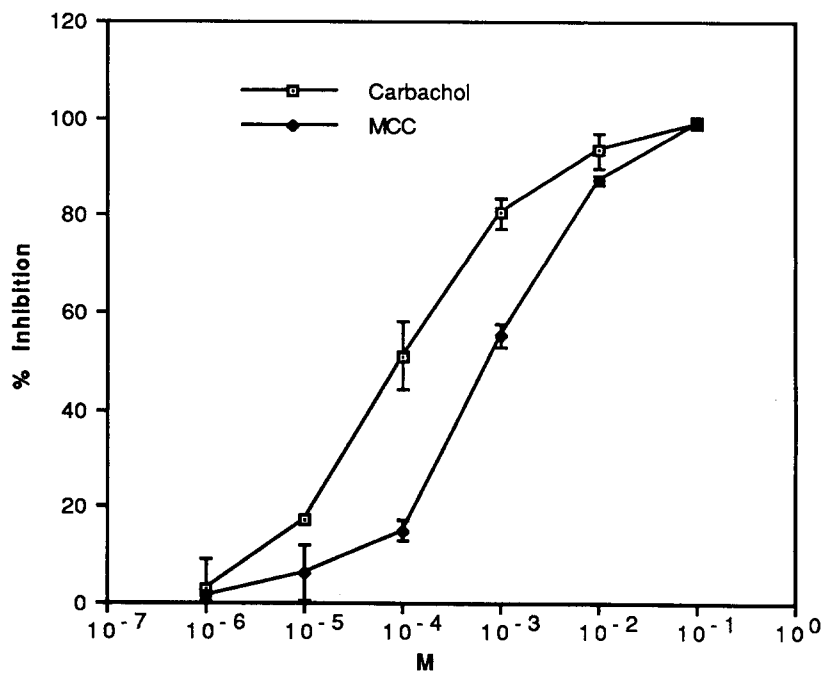

FIG. 7. Competition of [^{3}H]QNB binding by MCC and carbachol in M_1Y_1 cells.

and the M_1 transfected cells may be genetic in nature. Although there are slight differences in the sequence homology of the M_1 gene used in the present study (cloned from a mouse genomic library) and the rat brain M_1 gene, the similarity of the effects of MCC on [^{3}H]QNB binding and Pi turnover in mouse and rat brains would suggest that the structural difference alone cannot account for the difference in MCC's action on transfected cells and rodent brain. In the light of this discussion it would appear that the differential characteristics of MCC in brain and the M_1Y_1 cells is attributable to differences in the microenviroment of the transvected muscarinic receptors, affecting both receptor-tranduction efficiency and ligand selectivity.

ACKNOWLEDGEMENTS

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