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# Selectivity of McN-A-343 in Stimulating Phosphoinositide Hydrolysis Mediated by $M_1$ Muscarinic Receptors

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**SUMMARY**

The potency and efficacy of McN-A-343 (McN) in stimulating phosphoinositide (PI) hydrolysis were investigated in Chinese hamster ovary cells transfected with the m1 and m3 muscarinic receptor genes, in comparison with carbamylcholine (CBC). In m1 cells, CBC and McN increased PI hydrolysis by 17- and 9-fold over basal, respectively, with corresponding $EC_{50}$ values of 4.2 and 4.3 $\mu$M. Whereas the maximal stimulatory response to CBC was slightly less in m3 cells (11-fold over basal), McN elicited only up to a 2-fold increase in PI hydrolysis in these cells. Competition binding data with *N*-[$^3$H]methylscopolamine showed that McN had a higher affinity in m1 than in m3 cells, whereas CBC did not differentiate well between the two receptor subtypes. The partial agonistic activity of McN was demonstrated by its ability to suppress the stimulation by CBC to its own maximal response in both cell lines in a dose-dependent manner and by its low efficacy and the absence of receptor spareness. The PI response to the full agonist CBC in m3 cells was associated with a larger receptor reserve than in m1 cells. Thus, differences in receptor spareness cannot account for the apparent selectivity of McN in activating m1 muscarinic receptors. Differences in the sensitivity of m1 and m3 cells to McN were not due to differences in receptor concentration, despite the fact that the receptor density in m1 cells was 2-fold higher than in m3 cells. Our results suggest an actual selectivity (but not necessarily specificity) of the effects of McN in increasing PI hydrolysis mediated by $M_1$ receptors.

Pharmacologically distinguishable forms of the muscarinic acetylcholine receptor occur in different tissues and have been classified into $M_1$, $M_2$, and $M_3$ subtypes on the basis of the selectivity of novel antagonists (1). Using DNA-cloning techniques and sequence analysis of cDNAs, the genes corresponding to m1, m2, m3, m4, and m5 muscarinic receptor subtypes were identified (2–4) [using the nomenclature of Bonner *et al.* (4)]. Different subtypes of the muscarinic receptor mediate a variety of cellular responses, including inhibition of adenylate cyclase, increased breakdown of PI, and modulation of potassium channels, through coupling to G proteins (5). m1, m3, and m5 muscarinic receptors have been reported to couple to stimulation of PI hydrolysis (6, 7), whereas m2 and m4 receptors are linked mainly to inhibition of adenylate cyclase (6, 8, 9).

Although the number of muscarinic receptor subtype-selective antagonists has been growing steadily, there are only a few examples of subtype-selective agonists. Most of these agonists are reportedly selective for the $M_1$ receptor subtype, e.g., McN (10, 11), AF102B (12), and SR95639A (13). It is important to study the molecular basis for receptor subtype selectivity of these and other potential $M_1$-selective agonists, due to the potential therapeutic application of such a class of agonists. This is particularly important because this receptor subtype is most abundant in brain regions involved in memory (14). Although there are presynaptic deficits in cholinergic neurotransmission both in normal aging and in Alzheimer's disease (15, 16), there is evidence that the $M_1$ receptors (in aging and Alzheimer's disease) (16, 17) and PI hydrolysis (at least in normal aging) (18) are not affected. Thus, such selective agonists could be very beneficial as a replacement therapy to correct the deficiency in acetylcholine release, in order to improve memory without eliciting untoward peripheral effects.

McN [4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride] was selected as a prototype $M_1$-selective agonist in the present study. This unique agonist actually served as one of the earliest indicators of the possible existence of subtypes for muscarinic receptors (10). Different from other muscarinic agonists, McN activates ganglionic $M_1$-muscarinic receptors both *in vivo* and *in vitro* (11, 19), without significant cardiac effects (20), suggesting receptor subtype selectivity. However, an alternative hypothesis that has been advanced explains these observations according to the presence of tissue

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**ABBREVIATIONS:** PI, phosphoinositide; McN, McN-A-343; CBC carbamylcholine; CHO, Chinese hamster ovary; NMS, *N*-methylscopolamine; $E_{max}$, maximal response of agonist; $B_{max}$, maximal binding sites; $EC_{50}$, concentration of agonist required to elicit 50% of maximal response; KHB, Krebs-Henseleit buffer; PrBCM, propylbenzilylcholine mustard HCl; DMEM, Dulbecco's modified Eagle's medium; G protein, GTP-binding protein.

(instead of subtype) selectivity for McN (21). This tissue selectivity might result from differences in the efficiency of coupling of muscarinic receptors to the effector systems (22). This hypothesis is supported by the observation that McN is a weak partial agonist in activating PI hydrolysis in the brain (23, 24), although this response is mediated mainly, although not exclusively, by $M_1$-muscarinic receptors (24, 25).

In the present study we chose to compare the effects of McN in stimulating PI hydrolysis mediated by $M_1$- and $M_3$-muscarinic receptors, because both receptor subtypes are coupled to this response, at least in the cerebral cortex (24). Additionally, whereas $M_1$ receptors are important for memory (26), $M_3$ receptors mediate glandular secretion and smooth muscle contraction (27). CHO cells that have been induced to permanently express $M_1$- (28) or $M_3$- (29) muscarinic receptors were used, because of the advantage that each cell line represents a pure system that contains a single receptor subtype. Additionally, both cell lines share all properties except the subtype of the expressed receptor. It has been shown previously that the pharmacology of these expressed receptors corresponds to that of the $M_1$ and $M_3$ subtypes (30) and that activation of these receptors results in a robust increase in PI hydrolysis (6).

Therefore, the primary goal of this work was to elucidate whether McN actually differentiates between these two receptor subtypes and, if so, to investigate the molecular basis for such selectivity. The potential role of different possibilities that could account for the apparent receptor selectivity of McN was investigated, including the contribution of receptor reserve, receptor density, and binding selectivity of McN at the two receptor subtypes. Our results suggest actual selectivity (but not necessarily specificity) of the effects of McN in increasing PI hydrolysis mediated by $M_1$ receptors.

## Experimental Procedures

**Materials.** *myo*-[2-$^3$H]inositol and [$^3$H]NMS were from Amersham (Arlington Heights, IL). [$^{14}$C]Inositol-1-phosphate was from American Radiolabeled Chemicals Inc. (St. Louis, MO). CBC, atropine, and trypsin were from Sigma (St. Louis, MO). McN was from Research Biochemicals, Inc. (Natick, MA). PrBCM was from NEN products (Boston, MA). Tissue culture supplies were obtained from GIBCO (Grand Island, NY).

**Cell culture condition.** CHO cells stably transfected with restriction fragments of the m1 or m3 muscarinic receptor genes containing the entire coding regions were provided by Drs. J. C. Venter and C. M. Fraser at the National Institutes of Health (28, 29). The pharmacology of muscarinic receptors in these two cell lines is similar to that in analogous CHO cell lines established in other laboratories (30). Cells were grown in tissue culture flasks (75 cm$^2$, 250 ml) in 20 ml of DMEM supplemented with 10% (v/v) bovine calf serum and 0.005% (w/v) Geneticin. Cells were grown for 4–6 days at 37° in an atmosphere consisting of 10% $CO_2$ and 90% humidified air. Subculture was achieved using $D_1$ solution containing 0.05% trypsin (31). After centrifugation of the cell suspension for 1 min at 300 × *g*, the supernatant was removed and the cells were resuspended in DMEM and distributed into flasks (1:6) on day 0. The culture medium was changed on day 4 after subculture and every day thereafter by the addition of 10 ml of fresh DMEM and removal of 10 ml of medium. Cells were used for experiments 4–7 days after subculture.

**Assay of inositol phosphates release.** m1 and m3 CHO cells were harvested for assay as described above. The cells were then resuspended in 3 ml of KHB (in mM: NaCl, 118; KCl, 4.7; $KH_2PO_4$, 1.2; $MgSO_4$, 1.2; glucose, 1.2; $CaCl_2$, 1.3; $NaHCO_2$, 25) and incubated with 50 μCi of *myo*-[2-$^3$H]inositol for 1 h at 37°, with continuous bubbling with 95% $O_2$/5% $CO_2$. At the end of the labeling period, cells were suspended in 40 ml of fresh oxygenated KHB, followed by centrifugation in order to eliminate excess radioactivity. The cell pellet was suspended in KHB containing 10 mM LiCl and distributed into 10- × 75-mm glass test tubes (around 10,000 cells/tube for both cell lines). Agonists were then added and cells were incubated for 60 min at 37° with continuous oxygenation. The reaction was stopped with 1 ml of chloroform/methanol/concentrated HCl (2:1:0.01, v/v), and then 0.25 ml of chloroform and 0.25 ml of water containing about 1500 dpm of [$^{14}$C]inositol-1-phosphate were added to correct for losses during the isolation procedure. Then, 0.8 ml of the aqueous phase was loaded on Dowex AG2 X8 (formate form), followed by washing with 10 ml of water and 20 ml of 60 mM ammonium formate, 5 mM sodium borate. Total [$^3$H]inositol phosphates were eluted with 4 ml of 1.0 M ammonium formate, 0.1 M formic acid, and collected directly into scintillation vials. Ten milliliters of counting cocktail were added into each vial, and samples were counted for $^3$H and $^{14}$C and corrected individually for recovery and counting efficiencies. Data were calculated in terms of the response/$10^6$ cells. Results were expressed as a percentage of the maximal response to CBC in m1 cells, because experiments were performed in the two cell lines with both CBC and McN in parallel.

**Radioligand binding assays.** Cells were detached as described above and centrifuged at 300 × *g* for 1 min, followed by washing twice with KHB. For competition binding experiments, cells were then incubated for 60 min at 37° in 1 ml of KHB containing 0.2 nM [$^3$H]NMS and various concentrations of agonists, with continuous oxygenation. Nonspecific binding was defined in the presence of 2 μM atropine. For saturation measurements, a series of concentrations of [$^3$H]NMS ranging from 0.01 to 1 nM were used. The incubation was terminated by rapid filtration through Whatman GF/B filters, using a cell harvester, followed by rapid washing of the filters two times with 5 ml of ice-cold 0.9% NaCl solution. Each filter was then extracted in a scintillation vial in 4 ml of scintillation fluid for at least 6 hr before the radioactivity was determined by liquid scintillation counting, with automatic correction for counting efficiency.

**Receptor alkylation.** PrBCM was cyclized to the active aziridinium ion by incubation in 10 mM $Na^+/K^+$-phosphate buffer (pH 7.4) at 37° for 20 min. The cyclized PrBCM was added to the cell suspension at different concentrations according to the specific experimental designs. An equal volume of 10 mM $Na^+/K^+$-phosphate buffer was added to control cells. Cells were then incubated for 15 min at 37°, after which time the cells were washed three times with 15 ml of KHB by centrifugation at 300 × *g* for 1 min, to remove the unbound mustard. The cells were then resuspended in KHB for either PI turnover or radioligand binding experiments.

**Cell counts and protein determination.** Cell numbers were determined using a Coulter counter (model $Z_M$; Coulter Electronics). Protein content was assayed by the method of Lowry *et al.* (32).

**Data analysis.** Concentration-response data were fitted with a four-parameter logistic sigmoid model employing the program GraphPad (ISI, Philadelphia) to determine $EC_{50}$ and $E_{max}$ values. When appropriate, the inhibition constant ($K_B$) of McN in antagonizing CBC-induced PI hydrolysis was calculated by the Schild equation (33).

Agonist equilibrium dissociation constant ($K_A$) values were determined by the method of Furchgott (34). Equieffective concentrations of agonist in control ([*A*]) and PrBCM-treated ([*A'*]) cells were obtained graphically from the part of the dose-response curves encompassing 20–80% of the maximal response in the alkylated cells, and their reciprocals were plotted as described by Furchgott (34). $K_A$ was calculated from the equation:

$$K_A = (\text{slope} - 1)/\text{intercept}$$

Fractional receptor occupancy was calculated by:

$$\%\ \text{Occupancy} = 100\ ([A]/([A] + K_A))$$

The equilibrium dissociation constants ($K_A$) for agonists in inhibiting

the specific binding of 0.2 nM [$^3$H]NMS were estimated by the LIGAND program (35), using $K_D$ values for [$^3$H]NMS of 86 and 84 pM for m1 and m3 cells, respectively. Saturation experiments were analyzed by Rosenthal plots (36) and linear least-squares regression analysis using the concentration of unbound free ligand.

All values are represented as means ± standard errors. Student's $t$ test was employed for statistical analysis of the data. Statistical analysis was performed by comparing the values obtained in individual experiments.

## Results

**Dose-response curves of PI hydrolysis induced by CBC and McN in m1 and m3 CHO cells.** The concentration-response curves of CBC and McN in stimulating PI hydrolysis in m1 and m3 CHO cell lines were studied in parallel experiments and are shown in Fig. 1. In m1 cells, both CBC and McN increased PI hydrolysis in a dose-dependent manner, by 17- and 9-fold, respectively, with corresponding $EC_{50}$ values of 4.2 ± 0.8 μM and 4.3 ± 0.6 μM. The maximal effect ($E_{max}$) of CBC was 2-fold higher than that of McN ($P < 0.05$), without a significant difference in $EC_{50}$ (Fig. 1A). CBC exhibited a slightly higher potency ($EC_{50}$ = 2.0 ± 0.3 μM) but a lower $E_{max}$ (11-fold stimulation) in m3 cells (Fig. 1B). In contrast, McN induced only a 2-fold increase in PI hydrolysis, with an $EC_{50}$ of 10.2 ± 3.2 μM, in m3 cells (Fig. 1B).

**Antagonistic effects of McN in m1 and m3 cells.** Due to the partial agonistic nature of McN, we tested its ability to antagonize CBC-induced PI hydrolysis in m1 and m3 cells. Fig. 2 shows that McN inhibited CBC-induced PI hydrolysis in a dose-dependent manner in both m1 and m3 cells. In the presence of submaximal concentrations of CBC, McN further increased PI hydrolysis to its own maximal effect. On the other hand, when near-maximal CBC concentrations were used, McN inhibited the CBC responses so that they became equal to the maximal response of McN alone (Fig. 2). There was a close correspondence between the concentrations of McN required for its agonistic and antagonistic activity in both cell lines.

The dose-response curve of CBC in m3 cells was shifted to the right in a parallel fashion in the range of 3–10 μM McN (Fig. 3). At this concentration range, McN did not significantly depress the maximal response elicited by CBC or change the slope of the dose-response curve ($p > 0.05$). The resulting inhibition constant was 8.6 ± 1.6 μM. This value is similar to the $EC_{50}$ of McN-induced increase in PI hydrolysis in m3 cells (10.2 ± 3.2 μM). However, at higher concentrations (30–60 μM), McN significantly depressed both the maximal response and the slope of the dose-response curves of CBC ($p < 0.05$) (Fig. 3).

**Binding properties of CBC and McN in intact m1 and m3 CHO cells.** Competition curves for CBC and McN in inhibiting [$^3$H]NMS specific binding in m1 and m3 cells are shown in Fig. 4. CBC and McN competed for the specific binding of [$^3$H]NMS in a dose-dependent manner and with Hill slopes close to 1 (Table 1). Fitting of the competition data to a two-site model did not result in a statistically significant improvement of the fit, as compared with a one-site receptor model. Whereas CBC was twice as potent in m3 than in m1 cells, McN was 23-fold more potent in m1 than in m3 cells (Table 1).

**Effects of receptor alkylation on PI hydrolysis.** PrBCM was employed to irreversibly alkylate muscarinic receptors in m1 and m3 cells. After incubation with 200 nM PrBCM at 37° for 15 min, the maximal PI response to CBC in m1 and m3 cells was reduced to 50.4 ± 6.8% and 60.6 ± 6.8% of control, respectively (three experiments) (Fig. 5). The equilibrium dissociation constant ($K_A$) of CBC calculated by the method of Furchgott (34) was 44.1 ± 7.1 μM for m1 and 24.9 ± 4.3 μM for m3 cells. As shown in Table 1, these $K_A$ values are in excellent agreement with the $K_i$ values determined from the competition binding data.

In sharp contrast, when m1 cells were pretreated with only 10 nM PrBCM, the PI response to McN was reduced to 30.5 ± 4.8% of control values (three experiments) (Fig. 6). The calculated $K_A$ value of 6.2 ± 0.7 μM for McN was close to the $K_B$ value for the inhibition of the CBC-induced response in m1 cells (8.6 ± 1.6 μM). However, it was slightly higher than the $K_i$ value obtained in binding studies (2.0 ± 0.3 μM) (Table 1). It was not possible to calculate $K_A$ values for McN in m3 cells, due to the low magnitude of the PI response in this case.

**Relationship between the PI response and receptor occupancy.** When the fractional receptor occupancy by CBC

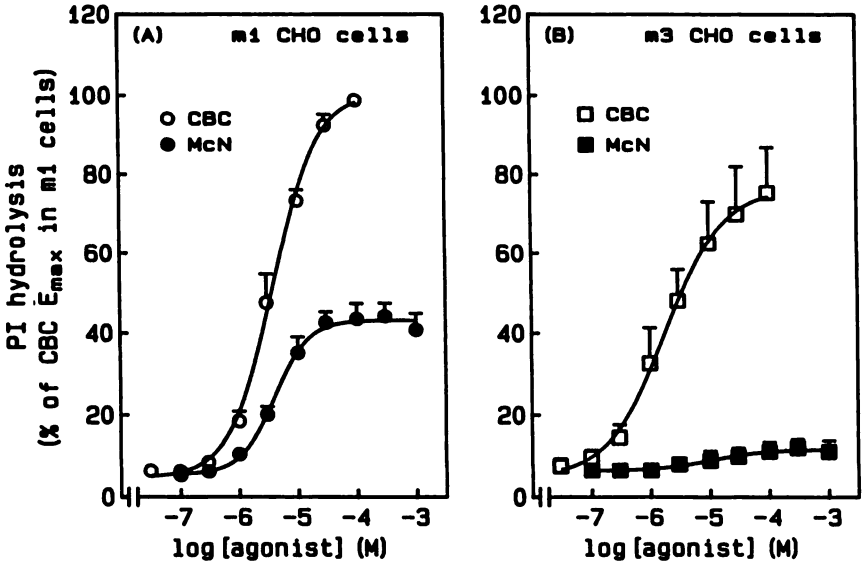


Fig. 1. Concentration-response curves for CBC and McN in m1 and m3 cell lines. Cells were incubated with increasing concentrations of either CBC (*open symbols*) or McN (*closed symbols*) for 60 min at 37°. The *curves* are the best fit from the computer-generated nonlinear least-squares regression analysis. The maximal response of CBC in m1 cells for each experiment was represented as 100% ($E_{max}$ = 2.0 ± 0.3 × $10^6$ dpm/$10^6$ cells above basal), because the experiments in the two cell lines were performed in parallel to compensate for day-to-day variability. Data shown are the means ± standard errors from three independent experiments, each determined in triplicate.

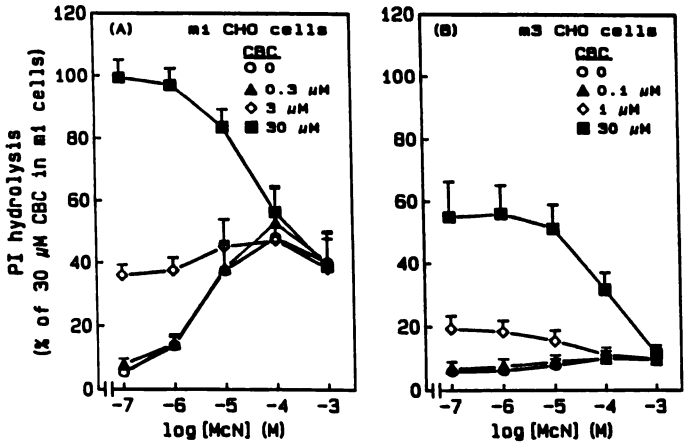


**Fig. 2.** Concentration-response relationship of the inhibition of CBC-induced PI hydrolysis by McN in m1 and m3 cells. Cells were preincubated with increasing concentrations of McN for 20 min at 37° and then stimulated with different concentrations of CBC for 60 min. The response of CBC at 30 μM in m1 cells was represented as 100% ($1.5 \pm 0.3 \times 10^6$ dpm/$10^6$ cells above basal). Data shown are the means ± standard errors from three independent experiments, each determined in triplicate, and the *curves* were drawn by connecting the data points.

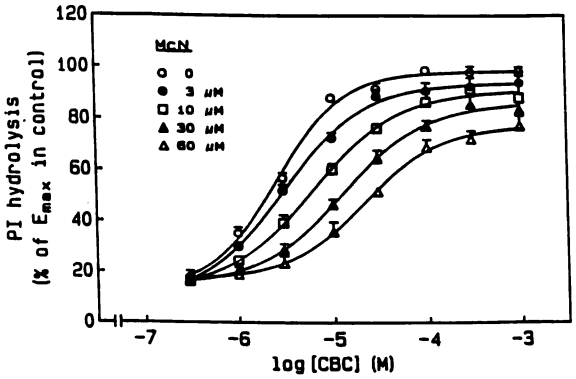


**Fig. 3.** Inhibition of CBC-induced PI hydrolysis by McN in m3 cells. The individual data points are shown as means ± standard errors of percentage of the maximal response of control cells, which was estimated by computer analysis. $E_{max}$ of the control group averaged $1.8 \pm 0.4 \times 10^6$ dpm/$10^6$ cells above basal. Each experiment was performed in triplicate.

(calculated using $K_A$) was compared with its dose-response curve in stimulating PI hydrolysis, the presence of spare receptors was observed in both m1 and m3 cell lines. Fig. 7 shows the relationship between the cellular response and occupancy of receptors. To reach a half-maximal increase in PI turnover, the required receptor occupancy was only 19.4 ± 6.6%of maximum for m1 (Fig. 7A) and 10.4 ± 1.9% for m3 cells (Fig. 7B), respectively. Additionally the $EC_{50}$ value for CBC in m1 cells was significantly lower (4.5-fold) than the corresponding $K_A$ value. This comparison in m3 cells also showed a more significant difference (9.2-fold) in the same direction, indicating the presence of a substantial receptor reserve for this response. It should be noted, however, that the observed shifts of the dose-response curves caused by 200 nM PrBCM in m1 and m3 cells (averaging 2.3 ± 0.3- and 4.7 ± 0.8-fold, respectively) are somewhat smaller than the expected shift calculated by the ratio of $K_A$ and $EC_{50}$ when all the spare receptors are inactivated to allow for depression of the maximal response (4.5- and 9.2-fold, respectively). The reasons for this discrepancy are not clear at present. However, we do not believe that this anomalous behavior of PrBCM results in erroneous interpretation of our data, because identical equilibrium dissociation constant values for CBC were obtained in receptor inactivation and radioligand binding experiments (Table 1).

In contrast, McN was shown to occupy a larger fraction of receptor sites in m1 cells to elicit its stimulation of PI hydrolysis (Fig. 8). Therefore, the percentage of maximal receptor occupancy required for a half-maximal response was 43.2 ± 1.7%, indicative of the presence of a very small receptor reserve, if any, for McN. Accordingly, the relative efficacy of McN was only 24% that of CBC in m1 cells. These findings provide an explanation for the higher sensitivity of McN-induced PI hydrolysis to receptor alkylation by PrBCM (Fig. 6).

**Relationship between receptor density and receptor subtype selectivity of McN.** Because m1 cells exhibited a higher muscarinic receptor density than m3 cells (Fig. 9), we decided to test whether this contributes to the apparent functional selectivity of McN. This was particularly important because it has been shown that there is an excellent correlation between maximal PI hydrolysis and muscarinic receptor density in murine fibroblast B82 cells that were transfected with the m1 receptor gene (37). Pretreatment of m1 cells with PrBCM resulted in a concentration-dependent decrease in muscarinic receptor binding sites (data not shown). At 2 nM, PrBCM reduced the density of muscarinic receptors in m1 cells to equal that present in m3 cells (Fig. 10A). However, a similar treatment failed to reduce the PI response to McN in m1 cells to equal that observed in m3 cells (Fig. 10B).

**Effects of various muscarinic receptor agonists on PI hydrolysis in m1 and m3 cells.** To further demonstrate the unique selectivity of McN for $M_1$ receptors, the effects of various muscarinic receptor agonists on PI hydrolysis were compared in m1 and m3 cells. As illustrated in Fig. 11, all of the agonists, except pilocarpine and McN, elicited full or near full stimulation of PI hydrolysis in both m1 and m3 cell lines at maximally effective concentrations. In contrast, pilocarpine and McN markedly discriminated between the two receptor subtypes (Fig. 11).

## Discussion

The pharmacological characteristics of CBC and McN in stimulating PI hydrolysis were investigated in CHO cells transfected with the m1 and m3 muscarinic receptor genes. The main points resulting from this work are 1) stimulation of m1 cells by CBC or McN increased PI turnover, for which the maximal effect of CBC was 2-fold higher than that of McN; 2) CBC increased PI hydrolysis by 11-fold in m3 cells, whereas McN was almost a pure antagonist in these cells; 3) McN possesses properties of a partial agonist, because it could antagonize the effects of CBC in both cell lines; 4) McN has a significantly higher binding affinity for the m1 than the m3 receptor subtype; and 5) our data indicate that the receptor subtype selectivity of McN in activating PI hydrolysis is not due to differences in the magnitude of receptor reserve or total muscarinic receptor density in the two cell lines.

McN served as one of the earliest tools used in identifying the existence of a unique $M_1$ muscarinic receptor subtype (10). The effects of McN in activating muscarinic receptors vary from one tissue to another. The initial reports demonstrated that McN exhibited the ability to selectively stimulate sympathetic ganglionic muscarinic receptors both *in vivo* and *in vitro*

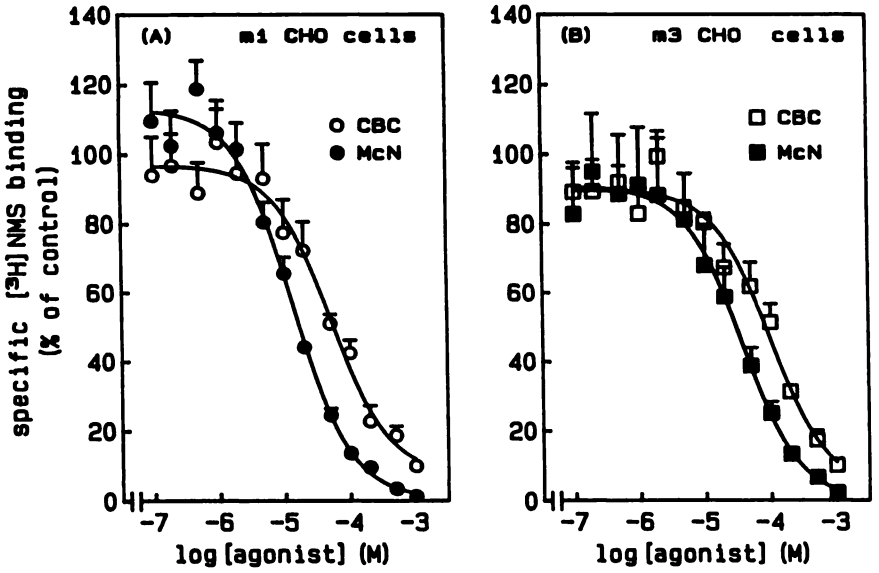


**Fig. 4.** Competition of CBC or McN for specific [$^3$H]NMS binding in m1 and m3 cells. Intact cells were incubated in triplicate with 0.2 nM [$^3$H]NMS, in the absence or presence of increasing concentrations of agonists. Data shown are the means ± standard errors of four independent experiments and are presented as percentage of specific binding obtained in the absence of agonists. The $K_i$ values for McN calculated using the LIGAND program were 2.0 ± 0.3 and 45.2 ± 11.7 μM in m1 and m3 cells, respectively. These values are significantly different ($p < 0.05$). The corresponding values for CBC were 53.4 ± 11.2 and 24.0 ± 3.3 μM (not significantly different from each other; $p > 0.05$). McN had a significantly higher affinity than CBC in m1 cells ($p < 0.05$). However, the affinities of the two agonists were not significantly different in m3 cells.

TABLE 1

**Parameters of CBC and McN in m1 and m3 CHO cells**

Values are represented as the means ± standard errors. The $K_i$ and Hill slope values are from competition binding experiments with 0.2 nM [$^3$H]NMS. The calculated $K_A$ and $EC_{50}$ values (in control cells) reported are derived specifically from the receptor alkylation experiments to provide a more accurate comparison between the two parameters.

| | $K_i$ | Hill slope | $K_A$ | $EC_{50}$ in control cells |
|---|---|---|---|---|
| | μM | | μM | μM |
| CBC | | | | |
| m1 | 53.4 ± 11.2 | 0.9 ± 0.1 | 44.1 ± 7.1 | 9.9 ± 2.4 |
| m3 | 24.0 ± 3.3 | 1.1 ± 0.1 | 24.9 ± 4.3 | 2.7 ± 0.2 |
| McN | | | | |
| m1 | 2.0 ± 0.3 | 0.9 ± 0.1 | 6.2 ± 0.7 | 4.7 ± 0.2 |
| m3 | 45.2 ± 11.7 | 1.0 ± 0.1 | | |

(11) and yet had little effect on muscarinic receptors present in either the heart or jejunum from dog, cat, and rabbit (20) or in rat ileum (38), suggesting receptor heterogeneity. This hypothesis was confirmed upon the discovery of the atypical selective antagonist pirenzepine (1), where $M_1$ receptors were characterized by a high affinity for this antagonist. Stimulation of ganglionic muscarinic receptors by McN was reversed by pirenzepine with high affinity (11). McN was proposed to be the first $M_1$-selective agonist. Later, several additional agonistic effects of McN on $M_1$ receptors were reported. McN significantly increased the amplitude of submaximal evoked electrical spikes in rat superior cervical ganglia via the activation of the $M_1$ receptors (39). It also enhanced stimulation-induced neurotransmitter release from guinea pig atria and ileum and rat cerebral cortex (40, 41) and induced inhibition of contraction in field-stimulated rabbit vas deferens (42) through the activation of the presynaptic $M_1$ receptors. Stimulatory effects of McN on $M_1$ receptors were also observed in the intramural neurons of rat duodenum (43). In contrast, McN exhibited no, or very weak, agonistic effects in tissues where muscarinic responses are not mediated by the $M_1$ receptor subtype, such as guinea pig ileum, bladder, heart, and trachea (44, 45).

However, other experimental evidence argues against the selectivity of McN for $M_1$ receptors. Black and Shankley (46) reported that McN stimulated gastric acid secretion, which is blocked by pirenzepine with low affinity. In guinea pig taenia caeci, McN acted as a full agonist (47). It was also interesting to find that McN has relatively little selectivity in its binding affinity for brain $M_1$ receptors versus cardiac $M_2$ receptors (44, 48). These observations casted doubt on the hypothesis that McN has selective affinity or intrinsic efficacy for $M_1$ receptors.

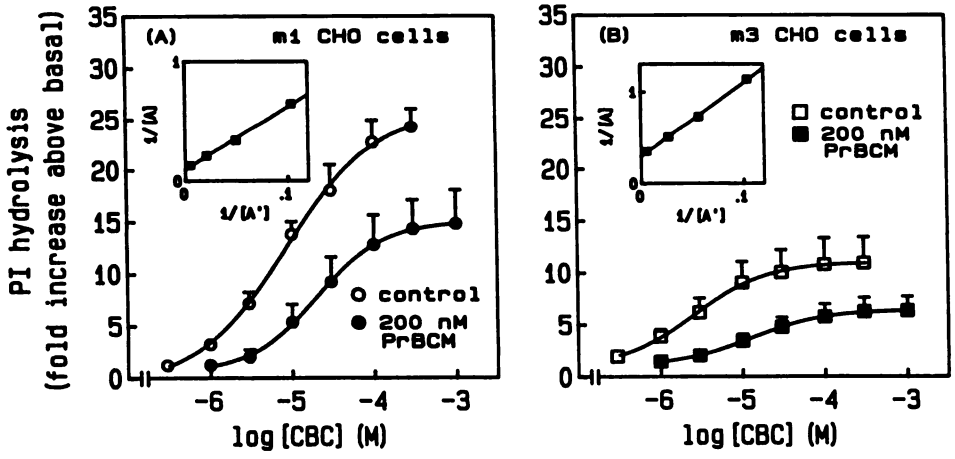


**Fig. 5.** Effects of PrBCM on CBC concentration-response curves for PI turnover in m1 and m3 cells. Cells were treated with either buffer or 200 nM cyclized PrBCM for 15 min at 37°, washed, and stimulated with CBC. Values shown are the means ± standard errors of three independent experiments and are presented as fold stimulation over basal PI hydrolysis. *Insets*, reciprocals of equieffective concentrations of CBC in control ([*A*]) and PrBCM-treated cells ([*A'*]) were plotted. $K_A$ values calculated by the method of Furchgott (34) were 44.1 ± 7.1 μM and 24.9 ± 4.3 μM for m1 and m3 receptors, respectively.

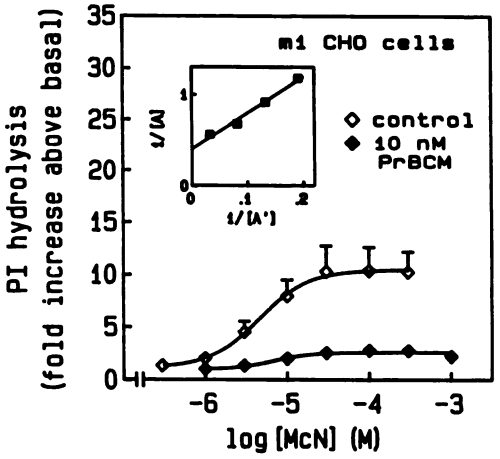


**Fig. 6.** Effects of PrBCM on concentration-response curves of McN-induced PI hydrolysis in m1 cells. Cells were treated with either buffer or 10 nM cyclized PrBCM for 15 min at 37°, washed, and stimulated with McN. Values shown are the means ± standard errors of three independent experiments and are presented as fold stimulation over basal PI hydrolysis. *Inset*, Furchgott's analysis of the data (see Experimental Procedures). $K_A$ values averaged 6.2 ± 0.7 μM.

An alternative hypothesis that emerged was that McN possesses tissue selectivity [i.e., tissue-selective *efficacy*, as defined by Stephenson (49), or *intrinsic activity*, coined by Ariens (50)] but not true receptor-selective *intrinsic efficacy* [as defined by Furchgott (34)]. Thus, being a partial agonist, McN would produce more pronounced agonistic activity either in tissues that have a higher receptor concentration or where muscarinic receptors are more efficiently coupled to intracellular transducers (21). In fact, there is evidence in the literature that the purported receptor subtype selectivity of some β-adrenergic receptor agonists might be a mere reflection of such differences in various tissues (51). Efficiency of coupling in different tissues also plays an important role in determining the apparent efficacy of partial muscarinic agonists. For example, Ringdahl (52) demonstrated that BM5 is a full agonist in guinea pig ileum and a weak partial agonist in the bladder; where contraction is mediated by the same muscarinic receptor subtype in both tissues but with a larger receptor reserve in the ileum. The contribution of the coupling efficiency to the apparent relative efficacy of muscarinic partial agonists is best shown by the observation that these agonists display variable relative efficacies in eliciting different responses in the same cell type (53).

In the present work, however, differences in receptor reserve do not appear to contribute to the higher maximal PI hydrolysis in response to McN in m1 cells. On the contrary, muscarinic receptors in m3 cells appear to be better coupled to PI hydrolysis than in m1 cells ($K_A/EC_{50}$ = 9.2 and 4.5 for CBC, respectively). Black and Leff (54) have proposed a model in which the efficacy of an agonist is related to the equilibrium dissociation constant of the agonist/activated receptor/G protein ternary complex. In this case, the relative efficacy of an agonist would vary from one tissue to another depending on the abundance of G proteins. This cannot explain the selectivity of McN observed in the present study, because both cell lines should express an equal concentration of endogenous G proteins, being derived from the same mother cells. However, we cannot discount the possibility that the McN-receptor complex might not be coupled to the same type of G protein in both cell lines.

Furthermore, differences in receptor concentration between m1 and m3 cells could not account for the apparent higher efficacy of McN in m1 cells. Thus, our data provide evidence that McN indeed possesses selective intrinsic efficacy in activating m1 versus m3 receptors. This selectivity of McN is supported by previous findings showing that it is a poor partial agonist in stimulating PI hydrolysis in 1321N1 human astrocytoma (55) and SK-N-SH human neuroblastoma (56) cells and in salivary glands.[2] These cell lines and tissues mainly express m3 muscarinic receptors (57, 58). This is in spite the fact that muscarinic receptors in these cells and tissues are very efficiently coupled to PI hydrolysis, as indicated by the magnitude of the maximal response to full muscarinic agonists and the large receptor reserve (59). Similar to McN, pilocarpine demonstrated preferential efficacy in m1 as compared with m3 cells. Interestingly, this partial agonist also shows significant selectivity in activating $M_1$ muscarinic receptor-mediated electrophysiological effects in the brain (6)) and is a very weak partial agonist in inducing $M_3$ receptor-mediated PI hydrolysis in SK-N-SH cells (61). In contrast, several compounds that are

[2] C. Forray and E. E. El-Fakahany, unpublished observations.

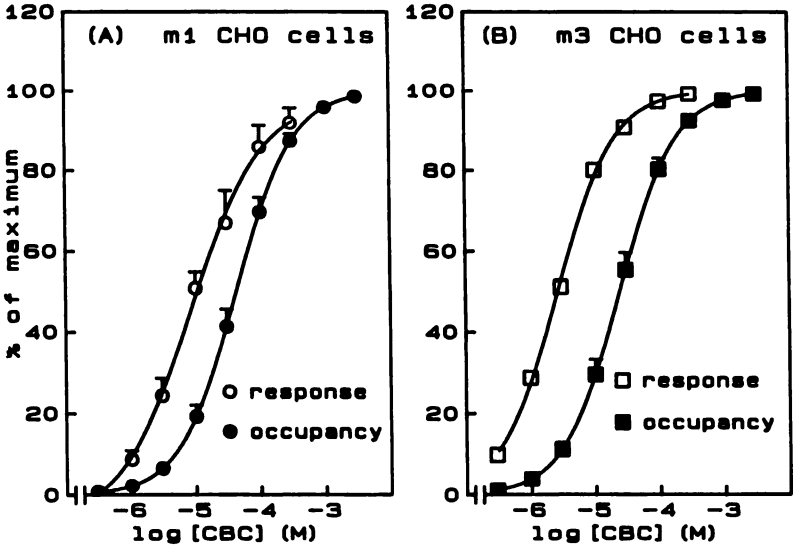


**Fig. 7.** Relationship between the PI response and receptor occupancy by CBC in m1 and m3 cells. Fractional receptor occupancy was calculated as described in Experimental Procedures, using the $K_A$ values derived from Furchgott's analysis. Response values shown are the means ± standard errors from three independent experiments and are presented as percentage of maximal response (2.8 ± 0.5 × $10^6$ dpm/$10^6$ cells for m1 and 0.7 ± 0.1 × $10^6$ dpm/$10^6$ cells for m3 cells) or maximal receptor occupancy. Data for both PI hydrolysis and receptor occupancy were derived from the same set of experiments.

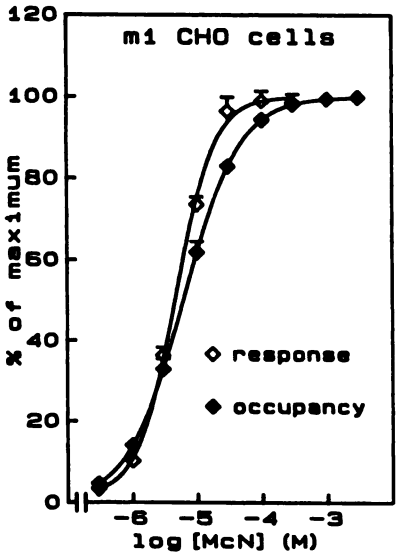


**Fig. 8.** Relationship between PI response and receptor occupancy by McN in m1 cells. Fractional receptor occupancy was calculated as described in the legend to Fig. 7. Values shown are the means ± standard errors from three independent experiments and are presented as percentage of maximal response (1.0 ± 0.3 × $10^6$ dpm/$10^6$ cells) or maximal receptor occupancy. Data for both PI hydrolysis and receptor occupancy were obtained from the same set of experiments.

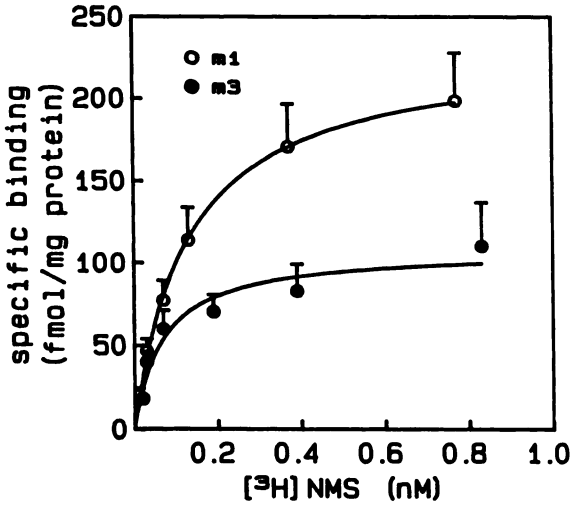


**Fig. 9.** Saturation isotherms of specific [$^3$H]NMS binding in m1 and m3 cells. Cells were incubated with a series of concentrations of [$^3$H]NMS at 37° for 60 min. Values shown are calculated in fmol/mg of protein from five to nine independent experiments. $B_{max}$ is 237.8 ± 34.6 fmol/mg of protein for m1 cells and 121.8 ± 31.2 fmol/mg of protein for m3 cells ($p < 0.05$) and the corresponding $K_D$ values were 154.7 ± 19.9 pM and 68.0 ± 3.0 pM.

partial agonists in increasing PI hydrolysis in the brain (bethanechol, arecoline, and oxotremorine) (23, 24) exhibited high apparent efficacy in both m1 and m3 cells. Interestingly, bethanechol is highly efficacious in stimulating PI hydrolysis in SK-N-SH cells (61). Thus, in the case of nonselective agonists, tissue-related efficacy, rather than selective intrinsic efficacy, plays an important role in determining the level of the maximal response (see below).

In addition to its selective efficacy, McN also showed a 23-fold higher affinity for m1 as compared with m3 receptors. This is different from CBC, which showed only a 2-fold difference. Both agonists bound to a single receptor conformation in both cell lines. This observation might be due to two main assay conditions; 1) binding was determined in intact cells, where the effects of endogenous GTP on agonist binding are pronounced (31, 62), and 2) prolonged exposure of intact cells to agonists at 37° results in significant alterations in agonist-receptor conformations (63).

However, McN still behaved as a partial agonist in both cell lines, with a much lower maximal response in m3 cells. This partial agonistic activity of McN was also indicated by its ability to suppress the stimulation of PI hydrolysis by CBC to its own maximal response in both m1 and m3 cell lines, in a dose-dependent manner. This antagonism was competitive, at least at 3–10 μM McN in m3 cells. However, higher concentrations of McN elicited apparent noncompetitive antagonism. We have no explanation for this latter phenomenon at present. In addition, the response to McN was much more sensitive than that of CBC to receptor alkylation (this work) or desensitization,[3] due to the lack of receptor spareness for McN.

The question remains as to why McN is a weak partial agonist in increasing PI hydrolysis (23, 24) and neuronal excitability (60) in the brain, although $M_1$ receptors are the major contributor to increasing PI hydrolysis in this tissue (24). In fact, the vast difference in the efficacy of McN in brain and ganglia has led to the speculation that there might be different subclasses of $M_1$ receptors ($M_{1\alpha}$ and $M_{1\beta}$) in the two tissues (64). This contention has actually been supported by the discovery of muscarinic antagonists that, different from pirenzepine, differentiate between the receptors in the two tissues (65). However, this hypothesis is currently debatable (66). Another possibility is that efficiency of coupling between $M_1$ receptors and transduction mechanisms varies among tissues, being very poor in the brain (24, 59) and high in sympathetic ganglia (21). This inefficient coupling in the brain would have a deleterious effect on the apparent efficacy of partial agonists such as McN. This is shown by its inability to differentiate between high and low agonist binding conformations in the brain, which reflect different states of coupling to G proteins (67). Thus, although McN has a selective intrinsic efficacy at $M_1$ receptors, the efficiency of coupling of these receptors in different tissues could determine its apparent agonistic activity. These conclusions are in accordance with the following model proposed by Kenakin (22), in which the response of a certain tissue to McN could be described by the following parameters:

$$\text{Response} = f(S) = f\{(\epsilon \cdot [R_t])/(1 + K_A/[A])\}$$

where $f$ is a function relating the stimulus ($S$) and response, $\epsilon$ is the intrinsic efficacy, $[R_t]$ is the receptor concentration, $K_A$ is the agonist-receptor equilibrium dissociation constant, and $[A]$ is the concentration of agonist. In this model, $f$ and $[R_t]$ are tissue-dependent factors, whereas $\epsilon$ and $K_A$ are dependent on the nature of the agonist.

In conclusion, McN, as a partial agonist, has agonistic selectivity for $M_1$ as compared with $M_3$ receptors. This subtype selectivity is not due to differences in the magnitude of receptor reserve or total muscarinic receptor density in the m1 and m3 cells, and it could be attributed to a true selective intrinsic efficacy. In addition, McN has a significantly higher binding affinity for m1 than m3 receptors. Efficient receptor-effector coupling must exist for this agonistic activity to become appar-

[3] J. Hu and E. E. El-Fakahany, Manuscript in preparation.

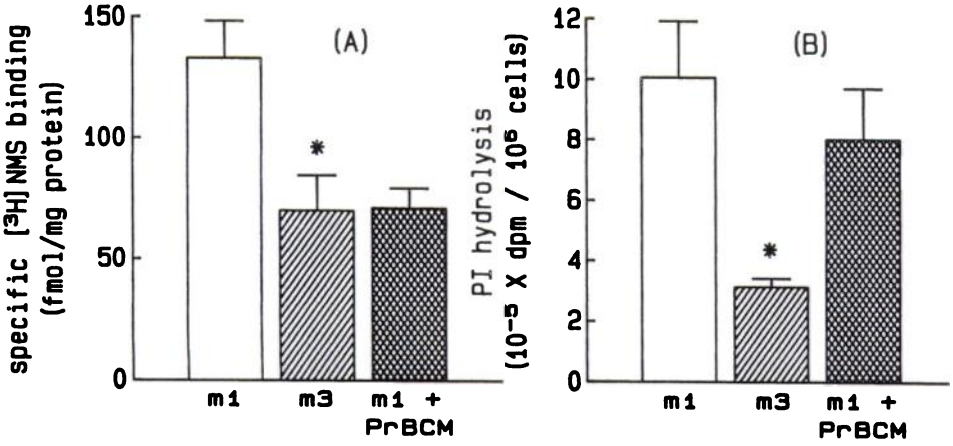


**Fig. 10.** Effects of PrBCM treatment on [$^3$H]NMS binding and McN-induced PI hydrolysis in m1 cells. m1 cells were preincubated with or without 2 nM PrBCM for 15 min at 37° and then washed. m1 or m3 cells were then incubated with 1 nM [$^3$H]NMS (for binding experiments) (A) or 1 mM McN (for PI hydrolysis) (B) for 60 min at 37°. *, Significantly different from the value in m1 cells, $p < 0.05$.

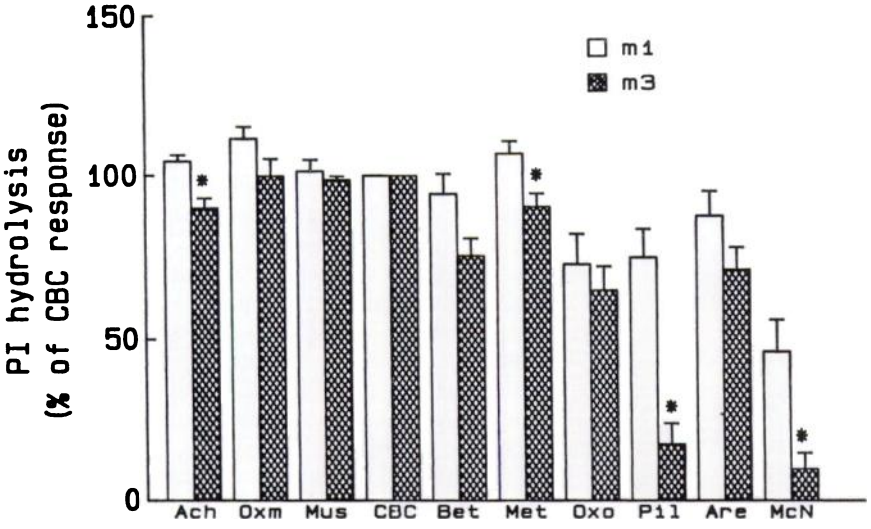


**Fig. 11.** Comparison of the maximal PI response to various muscarinic receptor agonists in m1 and m3 cells. Cells were pretreated with 10 μM diisopropylfluorophosphate and incubated with 1 mM of each agonist for 60 min at 37°. Data shown are the means ± standard errors from four independent experiments and are represented as percentage of the effect of CBC. *Ach*, acetylcholine; *Oxm*, oxotremorine-M; *Mus*, muscarine; *Bet*, bethanechol; *Met*, methacholine; *Oxo*, oxotremorine; *Pil*, pilocarpine; *Are*, arecoline. *, Significantly different from the value in m1 cells, $p < 0.05$.

ent. It should be stated, however, that, although McN exhibits selective intrinsic efficacy at $M_1$ receptors, it is by no means a specific agonist for these receptors. Thus, McN is known to inhibit adenylate cyclase in the heart and striatum (25), a response that is mediated by $M_2$ and $M_4$ receptors, respectively (9, 25). As stated above, it also causes contraction of some types of smooth muscle ($M_3$) (47). Thus, increasing the efficiency of coupling beyond a certain extent will reveal the agonistic activity of compounds with limited selective intrinsic efficacy.

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