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Interactions of Agonists with M2 and M4 Muscarinic Receptor Subtypes Mediating Cyclic AMP Inhibition

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SUMMARY

In this study the similarities and differences between the M2 and M4 subtypes in their recognition of agonists were explored. A CHO-K1 cell line transfected with the human m2 receptor was used as a homogeneous M2 tissue for comparison with two putative M4 systems (rat striatum and the N1E-115 mouse neuroblastoma cell line). The equilibrium binding dissociation constants and intrinsic efficacies for seven muscarinic agonists were determined for their stimulation of cyclic AMP inhibition via the M2 and M4 receptors. Partial receptor occlusion with propylbenzilylcholine mustard was used to determine binding constants for the more efficacious drugs and the reference agonist oxotremorine-M. The binding dissociation constants and relative efficacies for other agonists were then determined in reference to oxotremorine-M by a null method. For the M2 receptor the agonist binding dissociation constants ranged in potency from oxotremorine (1.5 μM) to bethanechol (171 μM), whereas relative efficacies varied from that of muscarine (relative efficacy = 0.9) to the value for McN-A343 (relative efficacy = 0.04). In general, most agonists bound with similar potencies to M2 and M4 receptors (K_d values within a factor of 2–3). However, oxotremorine bound to the N1E-115 and striatal M4 receptors about 3-fold and 10-fold less potently, respectively, than it did to the M2 receptor. Another exception was pilocarpine, which bound to the N1E-115 receptor (1.9 μM) with 8-fold and 12-fold higher potency than to the CHO-K1 M2 receptor and the striatal M4 receptor, respectively. Despite the low affinity of bethanechol for the M2 receptor, it was an efficacious agonist (maximal response equivalent to that of oxotremorine-M; relative efficacy = 0.6) at this subtype, whereas it was a partial agonist (60%) with lesser efficacy in the clonal M4 system. In contrast, McN-A343 and arecoline were significantly more efficacious at the two M4 receptors than they were at the M2 receptor.

The M4 system in the rat striatum displayed some similarity to the N1E-115 M4 system, with regard to the efficacy ranking for certain agonists (arecoline > bethanechol > McN-A343 ≥ pilocarpine). This rank order was different from the ranking of these four agonists in the M2 system, indicating that these two M4 receptors are more similar to each other in efficacy ranking than they are to the M2 receptor. However, the rat striatal and N1E-115 M4 receptors differed in their binding of oxotremorine and pilocarpine, indicating that these two M4 systems were not pharmacologically identical. In differentiating M2 and M4 receptors, the efficacies of bethanechol and McN-A343 and the binding potency of oxotremorine appear to be the most useful. Between N1E-115 and striatal M4 receptors, the binding of pilocarpine appears to be the primary distinguishing feature.

A current problem in muscarinic receptor pharmacology is the assignment of gene products to physiologically or biochemically identified muscarinic receptor-effector systems. Classically, selective antagonists have been the most useful ligands for the differentiation of receptor subtypes. With muscarinic receptors, however, antagonists are marginally selective at best; a combination of them should be used generally to identify the receptor subtype more confidently. Recently, we proposed that the muscarinic receptor that inhibits cyclic AMP levels in rat striatum is the m4 gene product (1). This conclusion was based in part on the knowledge that m4 but not m2 mRNA molecules are abundant in rat striatum (2) and on the unique (noncardiac) profile of antagonist potencies obtained from Schild analyses. Other investigators using muscarinic antagonists have also found a noncardiac profile for this receptor (3, 4). Further, the involvement of the m4 gene product is supported by the recent report of a large fraction of binding in striatum that was identified as M4, based on the similarity of antagonist binding potencies to those observed in a cell line containing the m4 receptor (5) and the fact that pilocarpine and arecoline display differing agonist intrinsic efficacies in striatum, compared with cardiac tissue (6).

The m2 and m4 primary sequences are more homologous to each other than they are to other muscarinic receptor subtypes and, of the five muscarinic receptor subtypes, the m2 and m4 gene products both couple efficiently to the inhibition of cyclic

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ABBREVIATIONS: M4, the pharmacologically defined receptor translated from the M4 gene transcript; EC_{50}, the concentration of agonist at which 50% of maximum response is elicited (a measure of agonist potency); K_d, equilibrium binding dissociation constant; M2, the pharmacologically defined receptor translated from the M2 gene transcript; PBS, phosphate-buffered saline; PGE_1, prostaglandin E_1.

AMP (7). Profiles of agonist intrinsic activities at muscarinic receptors mediating cyclic AMP inhibition in striatum (8) and in N1E-115 cells (9) differ in certain remarkable ways from the cortical M1 receptor mediating phosphoinositide turnover (10). Notably, tertiary amine agonists like arecoline, oxotremorine, and pilocarpine are weak at stimulating phosphoinositide turnover via the cortical M1 receptor but tend to have much more activity at receptors mediating cyclic AMP inhibition. Further, receptors in cortex and striatum inhibiting cyclic AMP appear to bind agonists with higher affinities than does the cortical M1 receptor mediating phosphoinositide turnover (11). Fundamental differences in agonist recognition, therefore, exist between muscarinic receptors differentially coupled to second messenger systems. In the present study, we examine whether differences in agonist recognition exist between muscarinic receptor subtypes (M2 and M4) that couple efficiently to the same effector system (adenylate cyclase inhibition).

In classical pharmacology, agonist binding constants and intrinsic efficacies are molecular properties of the ligand-receptor complex and as such should be suitable for differentiating receptor subtypes (12). Measurement of these properties of an agonist can be performed by a null method, which compares an agonist concentration-response curve with that of a more efficacious reference agonist (12, 13). The method allows calculation of both relative efficacy and binding dissociation constant for the drug if the binding dissociation constant for the reference agonist is known (see Experimental Procedures). In the present study, the reference agonist dissociation constants were determined by the method of partial receptor alkylation.

In the present study, we have evaluated agonist binding dissociation constants and relative efficacies at an M2 receptor and at two putative M4 receptors. Eight agonists were tested for cyclic AMP inhibition in CHO-K1 cells containing the human M2 receptor and in the rat striatum and the N1E-115 neuroblastoma cell line. The N1E-115 cell line contains the m4 transcript (14),[1] and recent pharmacological studies have shown that cyclic AMP inhibition in N1E-115 cells is of the noncardiac type (15, 16), implying involvement of the m4 gene product. The profiles of selective antagonist potencies are similar between the N1E-115 and striatal systems but differ significantly from potencies reported for these antagonists at cardiac M2 receptors (1, 16).

The present efficacy studies were accomplished by comparing the concentration-response curves for six drugs with those of reference agonists, oxotremorine-M and/or carbachol. The binding dissociation constants in N1E-115 cells for five of these agonists deduced from the null method correlated significantly ($r = 0.91$, $p < 0.05$) with binding dissociation constants for these drugs previously determined independently by other methods (9), providing strong evidence for the validity of the null method for deriving these constants at this receptor. Although there were some differences between the two M4 systems examined, they were more similar to each other than they were to the M2 system, with regard both to dissociation constants and to efficacies. The data indicate that certain partial agonists (arecoline, bethanechol, McN-A343, and pilocarpine) are recognized by M2 and M4 receptors with differing rank orders of relative intrinsic efficacies. However, there were certain differences between the two M4 receptors in these preparations, indicating nonidentity.

[1] M. McKinney and M. Robbins, unpublished data.

Experimental Procedures

Materials. Agonist drugs (oxotremorine, oxotremorine-M, carbachol, muscarine, arecoline, pilocarpine, bethanechol, and McN-A343) were obtained from either Sigma Chemical Co. (St. Louis, MO) or Research Biochemicals, Inc. (Wayland, MA). Propylbenzilylcholine mustard was obtained from DuPont/New England Nuclear. Fetal bovine serum was obtained from Clontech; other cell culture reagents were obtained from GIBCO. All other reagents were from Sigma.

Cell culture. Clone N1E-115 cells were grown without antibiotics under 10% CO_2, as described (9), in modified Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. N1E-115 cells were used after attainment of confluency (about 2 weeks after subculture). Chinese hamster ovary cells (CHO-K1) transfected with the human m2 receptor gene (CHO-2 cells) were obtained from Dr. M. Brann (National Institute of Mental Health) and cultured with the same conditions and medium as for N1E-115 cells, except with the addition of penicillin (1000 units/liter), streptomycin (1000 units/liter), and nonessential amino acids. For most assays with the CHO-2 line, cells were subcultured 2–4 days before the day of the assay and were detached typically at about 20% confluency. This constraint was imposed because we noted a substantial down-regulation of the M2 muscarinic receptor as confluency was attained with CHO-2 cells. N1E-115 cells were detached from 75-cm^2 flasks with Puck's D1 solution, collected by low speed centrifugation, washed, and then resuspended for assay in a physiological isoosmolar PBS (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose, 25 mM Na_2HPO_4; pH adjusted to 7.4 at 37°; osmolality adjusted to 340 ± 5 mOsm). CHO-2 cells were detached and treated similarly, except that the D1 solution contained 10 mM EDTA.

Tissue preparation and cyclic AMP assay. Male Sprague-Dawley rats (125–150 g) were fed *ad libitum* and sacrificed by decapitation. Dissociated striatal tissue was prepared as previously described (9, 11, 16, 17). In brief, the brain was rapidly dissected on an ice-cold aluminum block, and the striata were immersed in ice-cold PBS. The striata were minced and suspended in ice-cold D1 solution, and the mince was passed sequentially through Nytex 210- and 130-μm filters, using a glass rod. The filtrate was washed by centrifugation and resuspension in PBS (two times), and the dissociated cells were then metabolically labeled with 20–40 μCi of [^{3}H]adenine (22 Ci/mmol; Amersham) at 37° for 45 min. The ATP stores of N1E-115 cells and CHO-2 cells were labeled similarly. After radiolabeling of metabolic precursors, the labeling supernatant was removed and the cells were resuspended in PBS containing 1.5 mM isobutylmethylxanthine. After a 20-min preincubation with isobutylmethylxanthine, cells were stimulated with 10 μM forskolin (for assays of striatal and CHO-2 cells) or 1 μM PGE_1 (for assays of N1E-115 cells), in the presence or absence of various concentrations of muscarinic agonists, in 24-well culture plates, in a total volume of 400 μl, for 10 min at 37° in a shaking water bath. The reactions were stopped with the addition of 30 μl of 50% trichloroacetic acid. After the addition of ~2000 dpm/well [^{14}C]cAMP, which served as a "recovery standard," the radioactive cyclic AMP was purified on Dowex ion exchange columns as previously described.

Data analysis. After correction of the [^{3}H]cAMP product for recovery (using the [^{14}C]cAMP internal standard), the averaged value for basal (unstimulated) wells was subtracted from the levels found in the stimulated wells. The effect of muscarinic agonists was calculated as the percentage of inhibition of the forskolin- or PGE_1-elevated [^{3}H]cAMP level. Concentration-response curves were fitted with a four-parameter logistics model using the program ALLFIT (18), which provided the slope value (Hill coefficient), EC_{50}, and maximal response (percentage of inhibition) for each agonist. Generally, in every experiment the concentration-response curve for the agonist to be tested was obtained along with that for the reference agonist (usually oxotremorine-M, but occasionally carbachol). The parameters for each agonist

curve were obtained using seven to 10 different drug concentrations, each generally in triplicate or quadruplicate. To smooth out interexperimental variations in the reference agonist data, the computer-derived parameters for all experiments in each cell/tissue type were averaged. After normalization of the data to the reference agonist maximal response, the concentration-response curve for each of the other six agonists was then compared with the smoothed reference agonist(s) curve(s), derived from the averages of parameters from their individual curves.

The null method of deriving agonist relative intrinsic efficacies was discussed extensively by Kenakin (12). With this method, equieffective concentrations for a full (reference) and a second agonist are plotted as either double-inverted ($1/A_1$ versus $1/A_2$) or half-inverted (A_1 versus A_1/A_2) plots. The appropriate equation describing this method is derived as follows. The stimuli for the reference agonist (A_1) and the agonist to be compared (A_2) are expressed as

$$S_1 = \frac{E_1 \cdot R_t \cdot [A_1]}{[A_1] + K_1} \qquad S_2 = \frac{E_2 \cdot R_t \cdot [A_2]}{[A_2] + K_2}$$

where E_1 and E_2 are the efficacies of the two drugs, A_1 and A_2 are their concentrations, K_1 and K_2 are their equilibrium binding dissociation constants, and R_t is the total number of receptors in the tissue.

At equieffective concentrations, $S_1 = S_2$, the parts on the right of the equations given above can be set equal, and the resulting equation can be rearranged to the following:

$$[A_1] = \frac{K_1}{(E_1/E_2) - 1} + \frac{K_2}{(E_2/E_1) - 1} \cdot \frac{[A_1]}{[A_2]}$$

A plot of A_1 versus A_1/A_2 will produce a straight line with a slope equal to $K_2/(E_2/E_1 - 1)$ and a y-intercept equal to $K_1/(E_1/E_2 - 1)$. If the binding dissociation constant for one of the agonists is known (K_1), the parameters from this straight line can be used to calculate the relative efficacy (E_2/E_1) and the dissociation constant of the other agonist (K_2). The slope of this straight line will be negative when the second agonist is less efficacious than the first.

As pointed out by Kenakin (12), this null method for efficacy determination works best for agonists with a substantial difference in intrinsic efficacies. Thus, for our studies we chose the most efficacious agonist possible to use as the reference agonist; this was oxotremorine-M. The binding dissociation constant for oxotremorine-M in each of the cell preparations was determined by the method of Furchgott and Burnstyn (19) after partial receptor alkylation, using propylbenzilyl choline mustard, following our previously described protocol (11). We chose this method of obtaining the binding constant rather than radioligand binding because agonists generally bind to muscarinic receptor populations with multiple affinities, and certain assumptions must be made regarding which of these affinities reflect the active conformation. The method of Furchgott and Burnstyn (19), however, obtains a binding constant for the agonist in a "functional" assay system in which the agonist-receptor complex is known to be that which is the active conformation (i.e., the method obtains the K_d in the assay in which the complex is mediating a response). In the case of the rat striatum and N1E-115 cells, the binding dissociation constant for carbachol had been previously determined by partial receptor inactivation (11, 20); these values were 2.9 and 13 μM, respectively. Thus, we also used carbachol as a reference agonist in these two systems. In general, the K_d values for other agonists (arecoline, etc.) derived from the two reference agonists were in good agreement and were pooled to obtain the average value for each agonist in each tissue.

In this study, as in our previous studies (11, 20), Furchgott plots were constructed by determining the equieffective agonist concentrations (A and A') from the fitted curves for the "control" and "treated" data. That is, the four-parameter logistics model (using ALLFIT) was used to fit the two sets of concentration-response curves and then the "best-fit" parameters were used to calculate A and A' values over a response range in the upper half of the depressed (treated) curve. The particular value of the binding dissociation constant that is derived from the Furchgott plot is dependent on the particular range of responses chosen in the determination of A and A' values (12). Thus, to ensure consistency between independent experiments in the determination of these A and A' values, a computer program was used to derive these values from the parameters for the fitted curves over the response range of 60–85% of the maximal response determined for the treated curve. Because these fitted curves result from best fits using all the data in a given experiment (i.e., all points throughout the concentration-response curve), the dissociation constant thus calculated from the Furchgott plot was the best value obtainable from the entire set of data comprising the individual experiment. The dissociation constants calculated in these independent experiments were then averaged and the standard error of the mean was determined.

In the calculation of relative efficacies we found that, with the large number of reference curves that were accumulated, we could eliminate a good deal of experimental variation by comparing individual agonist curves with an averaged reference curve. For this, the curve-fitting program was used to determine the parameters (EC_{50} value, Hill slope, and maximal inhibition) for each of the 17–28 oxotremorine-M curves in each tissue. These parameter values were then averaged and used to generate the "smoothed" reference curves, and it was with these curves that comparisons were made, using a spreadsheet program.

Many of the agonists used in the present study were less efficacious than oxotremorine-M. However, in some experiments agonists (e.g., arecoline or oxotremorine) with a relative intrinsic activity of 0.9 or greater might produce an A_1 versus A_1/A_2 plot with a positive slope, indicating a reversed order of efficacy (i.e., in that experiment the agonist possessed more efficacy than oxotremorine-M). Examination of the equation shows that in such cases A_2 can be plotted versus A_2/A_1 to obtain a negative slope value from which the intrinsic efficacy can be obtained. The binding constants for the agonists that were close in efficacy to oxotremorine-M were independently determined by the partial receptor occlusion method.

For the case in which an agonist with substantially less intrinsic activity than oxotremorine-M or carbachol was evaluated, the value E_2/E_1 is negligibly low, and the K_2 approximates the magnitude of the slope of the A_1 versus A_1/A_2 plot (12, 13). Thus, even though the binding dissociation constant (K_1) for carbachol was not obtained in CHO-2 cells, the approximate K_2 values for agonists like McN-A343 and pilocarpine could be derived because of the large difference in relative intrinsic activities for the latter agonists, compared with carbachol.

Results

Determination of the binding dissociation constants for the reference agonist oxotremorine-M. The method of determining the relative intrinsic efficacy works best when there is a large difference in efficacies between the reference agonist and the agonist to be compared. For N1E-115 cells and for rat striatum, the binding dissociation constants for the efficacious agonist carbachol were previously determined by the method of partial receptor occlusion (11, 20). For the present study, oxotremorine-M was also used as a reference agonist. Both carbachol and oxotremorine-M were thus used to determine intrinsic efficacies and agonist binding dissociation constants in two of the three systems studied.

Figs. 1–3 show examples of the Furchgott method (19) as applied to the N1E-115 cells, the rat striatum, and the CHO-2 cells, respectively, for determination of the binding dissociation constant for oxotremorine-M. The alkylating agent used was propylbenzilylcholine mustard, following our previously described protocol (11). In the example shown in Fig. 1, the equilibrium binding dissociation constant in N1E-115 cells was 0.83 μM; the average of three experiments of this type was 1.39 $\pm$ 0.2 μM (Table 1). In the striatum (an example of one experiment is shown in Fig. 2; K_d = 1.78 μM), the average of seven

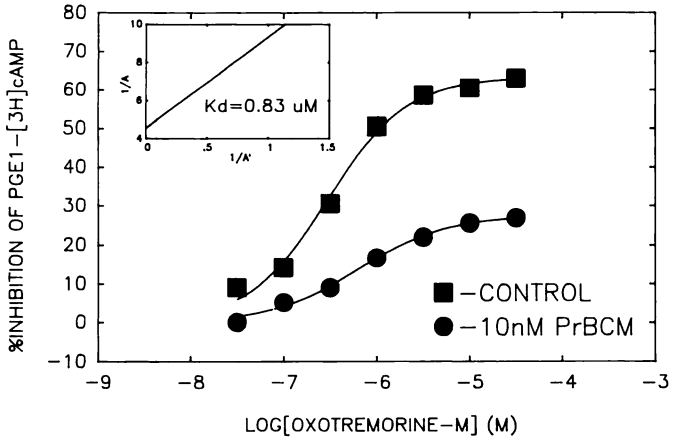

Fig. 1. Determination of binding dissociation constant for oxotremorine-M by the method of partial receptor occlusion in N1E-115 cells. The response curve for the agonist obtained with cells treated with 10 nM propybenzilylcholine mustard (●) was compared with that of untreated cells (■) in the same assay. *Inset*, K_d value obtained (0.83 μM) using Furchgott's method (19); the *line* shown was computer derived from the fitted concentration-response curves. Three experiments with N1E-115 cells were performed.

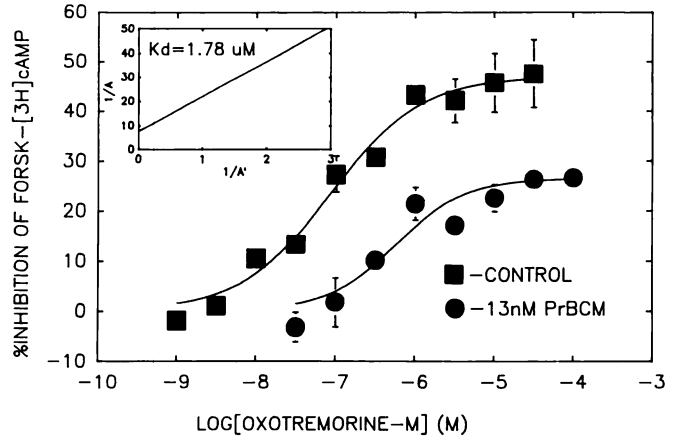

Fig. 2. Determination of binding dissociation constant for oxotremorine-M by the method of partial receptor occlusion in rat striatum. Dissociated cells were treated with 13 nM propylbenzilylcholine mustard (●) or buffer (■); after wash out of excess drug the concentration-response curves were obtained. The plot in the *inset* (Ref. 19) gave a K_d value of 1.78 μM for oxotremorine-M; the *line* shown was computer derived from the fitted concentration-response curves. This is one of seven similar experiments with rat striatal tissue.

experiments was 1.23 ± 0.2 μM (Table 2), a value not significantly different from that obtained in N1E-115 cells. In CHO-2 cells the binding dissociation constant for oxotremorine-M was somewhat higher; Fig. 3 shows an experiment for which a K_d value of 5.16 μM was obtained. For five experiments with CHO-2 cells, the average ± standard error value was 3.3 ± 0.5 μM (Table 3).

Determination of relative intrinsic efficacies for seven agonists in the M2 and putative M4 systems. Concentration-response experiments were performed in N1E-115 cells, dissociated rat striatum, and CHO-2 cells, with the reference agonist and the other six agonists. Generally, in each experiment a curve for the reference agonist (oxotremorine-M and/or carbachol) was included and the data were normalized to the estimated (by curve-fitting) or measured (at 100 μM concentration) maximal response of the reference agonist. Figs. 4–6 show

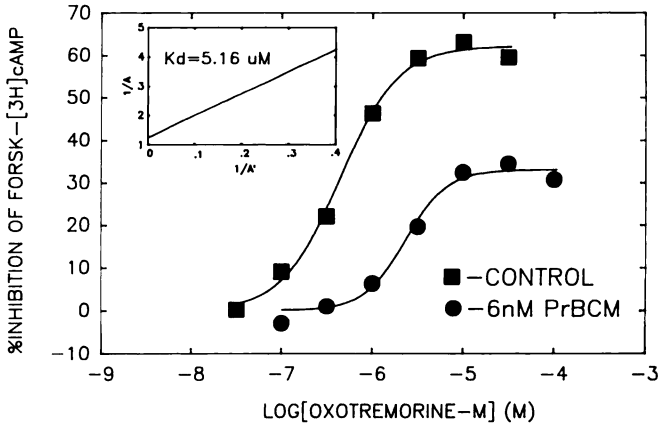

Fig. 3. Determination of the binding dissociation constant for oxotremorine-M by the method of partial receptor occlusion in CHO-2 cells. Cells in suspension were treated with 6 nM propylbenzilylcholine mustard (●) or buffer (■), as described in Experimental Procedures. *Inset*, Furchgott plot (19) for the data, with which a K_d value of 5.16 nM was obtained; the *line* shown was computer derived from the smoothed concentration-response curves. This is one of five similar experiments with CHO-2 cells.

composites of all normalized data for the three tissues. Table 4 contains the averaged EC_{50} values, Hill slopes, and maximal values (oxotremorine-M maximum = 100) for the individual experiments. Oxotremorine-M, carbachol, oxotremorine, and muscarine appear to be full, or nearly full, agonists in all three tissues. Arecoline was a full agonist in N1E-115 cells and the striatum but displayed an intrinsic activity of 88% in the CHO-2 cells. Similarly, the partial agonist McN-A343 had somewhat more activity in N1E-115 cells and striatum than in CHO-2 cells. Conversely, bethanechol was a full agonist in CHO-2 cells but a partial agonist in the other two tissues. The efficiency of coupling or degree of "spareness" in these three receptor systems can be described by calculating the ratio of the EC_{50} and K_d values for oxotremorine-M. These ratios were 3.5, 8.8, and 6.6 for the N1E-115 M4 receptor, the striatal M4 receptor, and the CHO-2 M2 receptor, respectively. Thus, of the three receptor systems, the rat striatal M4 receptor displayed the highest "efficiency" of coupling.

The relative intrinsic efficacies of six agonists were determined by comparing concentration-response curves with those of the reference agonists oxotremorine-M and/or carbachol. Because binding dissociation constants for carbachol had been previously determined at the M4 receptors (they were 2.9 and 13 μM in striatum and N1E-115 cells, respectively), it was also used as a reference agonist. Its efficacy appeared to be nearly equal to that of oxotremorine-M, judging from the relative efficacies that were obtained using it (data not shown), which were generally close in value to those obtained using oxotremorine-M. Using the data in Table 4 for the concentration-response curves for carbachol and oxotremorine-M and the K_d values for these agonists determined by partial receptor occlusion, the equation $E_2/E_1 = P_1/P_2$ (i.e., that relative efficacies are inversely proportional to occupancies required to mediate equal responses) was used to calculate the relative efficacy of carbachol in N1E-115 cells as 1.1 and in striatum as 0.64. Using both oxotremorine-M and carbachol as reference agonists about doubled the number of estimates of the binding dissociation constants in N1E-115 cells and striatum.

Tables 1–3 present the binding dissociation constants and

TABLE 1

Binding dissociation constants and relative efficacies for seven agonists for the inhibition of cyclic AMP levels in N1E-115 neuroblastoma cells

The binding dissociation constant is the concentration of drug at which half-maximal occupancy of the receptor occurs. *n*, number of independent experiments performed.

Agonist	n	Dissociation constant		n	Relative efficacy[a]	P_1/P_2[b]
		From relative efficacy	From Ref. 9			
		μM				
Oxotremorine-M	3	1.39 ± 0.2[c]		17	1.0	
Muscarine	6	6.2 ± 0.7		3	0.55 ± 0.07	0.77
Arecoline	6	10.8 ± 1.5	13	3	0.62 ± 0.07[d,e]	0.59
Bethanechol	10	132.5 ± 8[e]	76	6	0.29 ± 0.05[d,f]	0.35
McN-A343	8	11.8 ± 1.4[e]	7	4	0.24 ± 0.03[d,f]	0.25
Oxotremorine	11	5 ± 0.7[d,e]	1.7	7	0.82 ± 0.05[f]	1.67
Pilocarpine	8	1.9 ± 0.2[d,e]	3.4	4	0.17 ± 0.03[d,f]	0.18

[a] Within the column, all the values are significantly different from the value of the reference agonist; the values for muscarine, arecoline, and oxotremorine are not significantly different from each other but are significantly different from the efficacies of bethanechol and McN-A343. Level of significance, at least $p < 0.05$.
[b] Calculated using the K_d value in column 3 and the dose-response values in Table 4.
[c] Binding constant determined by the method of Furchgott and Burnstyn (19).
[d] Significantly different from the value for the agonist in CHO-2 cells, in Table 3, $p < 0.05$.
[e] Significantly different from the value for the agonist in striatum, in Table 2, $p < 0.05$.
[f] Significantly different from oxotremarine-m efficacy, $p < 0.05$.

TABLE 2

Binding dissociation constants and relative efficacies for seven agonists for the inhibition of cyclic AMP levels in rat striatum

The binding dissociation constant is the concentration of drug at which half-maximal occupancy of the receptor occurs. *n*, number of independent experiments performed.

Agonist	n	Dissociation constant	n	Relative efficacy[a]	P_1/P_2[a]
		μM			
Oxotremorine-M	7	1.23 ± 0.2[b]	28	1.0	
Muscarine	3	4.41 ± 1.3[b]	3	0.86 ± 0.37	0.83
Arecoline	4	4.3 ± 0.4[b]	3	1.14 ± 0.12[c,d]	0.83
Bethanechol	14	83 ± 5[c,d]	8	0.41 ± 0.12[e]	0.19
McN-A343	8	4.3 ± 0.3[c,d]	4	0.23 ± 0.06[c,e]	0.19
Oxotremorine	6	16.4 ± 2.3[b,c,d]	3	1.23 ± 0.69	10
Pilocarpine	8	22.7 ± 3.3[d]	4	0.11 ± 0.01[e]	0.10

[a] Calculated using the K_d value in column 3 and the dose-response values in Table 4.
[b] Binding constant determined by the method of Furchgott and Burnstyn (19).
[c] Significantly different from the value for the agonist in CHO-2 cells in Table 3, $p < 0.05$.
[d] Significantly different from the value for the agonist in N1E-115 cells in Table 1, $p < 0.05$.
[e] Significantly different from oxotremorine-M efficacy, $p < 0.05$.

relative efficacies obtained in N1E-115 cells, striatum, and CHO-2 cells using this method. Previously, in N1E-115 cells the binding dissociation constants for arecoline, bethanechol, oxotremorine, pilocarpine, and McN-A343 were determined by more direct methods (7). Those data (which are shown in column 4 of Table 1) thus can provide an independent check on the method used in this study. When the binding dissociation constants in Table 1, column 3, for these agonists were compared with those in Table 1, column 4 (from Ref. 9), using linear regression, a significant correlation coefficient of 0.908 was obtained ($p < 0.05$). The slope of this line was near unity (0.98) and passed close to the origin, indicating strong similarity between the data. These results thus provide support for the use of the null method for deriving binding dissociation constants. Using the agonist/reference agonist null method in the striatum (Table 2), it was not possible to derive accurate K_d values for muscarine, arecoline, or oxotremorine due to the high efficacies of these agonists. The K_d values shown for these drugs in the striatum, and for muscarine in the CHO-2 cells (Table 3), were obtained by the method of partial receptor occlusion.

As the extensive footnotes in Tables 1–3 indicate, the analysis of similarities and differences between M4 systems and between the M4 and M2 systems was quite involved. Several observations can be made, however, to summarize these studies.

TABLE 3

Binding dissociation constants and relative efficacies for seven agonists for the inhibition of cyclic AMP levels in CHO-2 cells

The binding dissociation constant is the concentration of drug at which half-maximal occupancy of the receptor occurs. *n*, number of independent experiments performed.

Agonist	n	Dissociation constant	n	Relative efficacy[a]	P_1/P_2[a]
		μM			
Oxotremorine-M	5	3.3 ± 0.5[b]	27	1.0	
Muscarine	6	16 ± 3[b]	6	0.85 ± 0.15	0.29
Arecoline	4	5 ± 0.8	4	0.29 ± 0.04[c,d,e]	0.39
Bechanechol	13	171 ± 17[c]	7	0.58 ± 0.06[d,e]	1.67
McN-A343	10	22.5 ± 1.8[c]	5	0.04 ± 0.003[c,d,e]	0.04
Oxotremorine	6	1.51 ± 0.3[c,d]	8	0.63 ± 0.22	1.11
Pilocarpine	10	15.9 ± 1.7[d]	5	0.09 ± 0.01[d,e]	0.08

[a] Calculated from the K_d value in column 3 and the dose-response values in Table 4.
[b] Binding constant determined by the method of Furchgott and Burnstyn (19).
[c] Significantly different from the value for the agonist in striatum, in Table 2, $p < 0.05$.
[d] Significantly different from the value for the agonist in N1E-115 cells, in Table 1, $p < 0.05$.
[e] Significantly different from oxotremorine-M efficacy, $p < 0.05$.

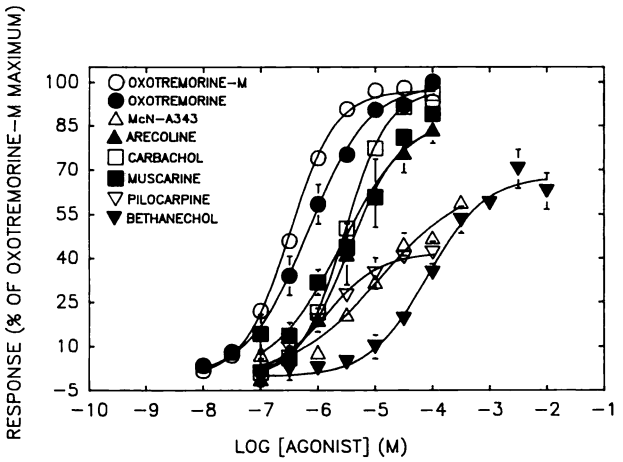

Fig. 4. Composites of concentration-response data for eight agonists in N1E-115 cells for the inhibition of 1 μM PGE_1-induced cyclic AMP levels. The responses of all agonists were normalized to the maximal response of oxotremorine-M (or carbachol in a few experiments) and averaged at each drug concentration. Table 4 contains the number of independent experiments (4–17) and the averaged parameter values for each agonist.

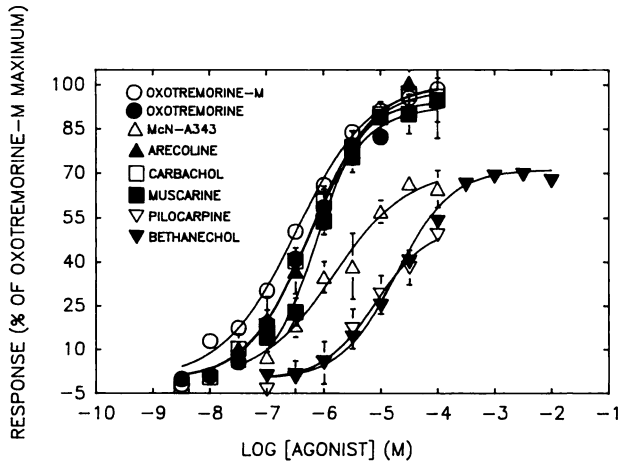

Fig. 5. Composite of concentration-response data for eight agonists in rat striatum for the inhibition of 10 μM forskolin-induced cyclic AMP levels. The responses of all agonists were normalized to the maximal response of oxotremorine-M (or carbachol in a few experiments) and averaged at each drug concentration. Table 4 contains the number of independent experiments (3–28) and the averaged parameter values for each agonist.

With regard to agonist binding potencies, most of the drugs did not differ by more than 2–3-fold in the three receptor systems. Oxotremorine, however, bound to the M2 receptor with higher affinity than to either of the two M4 systems. In the M2 system in the CHO-2 cells, both arecoline and oxotremorine tended to bind more potently than muscarine and McN-A343, whereas in both the M4 systems these four agonists were more nearly equal in binding potency. Notably, pilocarpine bound to the N1E-115 M4 receptor (1.9 μM) (Table 1) with 12-fold greater affinity than to the striatal M4 receptor (22.7 μM) (Table 2) and with 8-fold more affinity than to the CHO M2 receptor (15.6 μM) (Table 3). The binding dissociation constant for pilocarpine was so different between N1E-115 cells (1.9 μM) and striatum (22.7 μM) that we confirmed the value in the latter tissue by the method of partial receptor occlusion. A value of 18 ± 2 μM (three experiments) was obtained with this method, and this value was not significantly different from the

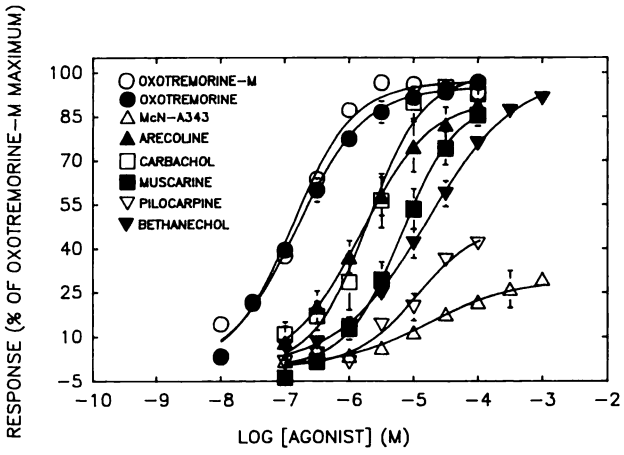

Fig. 6. Composite of concentration-response data for eight agonists in CHO-2 cells for the inhibition of 10 μM forskolin-induced cyclic AMP levels. The responses of all agonists were normalized to the maximal response of oxotremorine-M and averaged at each drug concentration. Table 4 contains the number of independent experiments (4–27) and the averaged parameter values for each agonist.

TABLE 4

Pharmacological data (EC_{50} values, Hill slopes, and maximal responses relative to oxotremorine-M maximum = 100%) for eight agonists in three tissues for mediation of cyclic AMP inhibition

n, number of independent experiments performed.

Agonist	*n*	EC_{50}	Hill slope	Maximal response
		μM		%
N1E-115 cells				
Oxotremorine-M	17	0.4 ± 0.18	1.06 ± 0.04	100 ± 4
Carbachol	8	3.1 ± 0.19	1.09 ± 0.09	101 ± 3
Oxotremorine	7	0.8 ± 0.22	1.02 ± 0.05	96 ± 2
Muscarine	3	2.9 ± 0.8	0.71 ± 0.09	91 ± 3
Arecoline	3	5.9 ± 3.44	0.85 ± 0.06	92 ± 2
Bethanechol	6	66 ± 14.4	0.95 ± 0.13	60 ± 7
McN-A343	4	9.3 ± 3.7	0.8 ± 0.19	58 ± 4
Pilocarpine	4	1.4 ± 0.28	1.08 ± 0.23	42 ± 4
Rat striatum				
Oxotremorine-M	28	0.14 ± 0.11	0.79 ± 0.04	100 ± 3
Carbachol	10	0.55 ± 0.03	0.82 ± 0.06	99 ± 1
Oxotremorine	3	0.17 ± 0.22	0.74 ± 0.07	95 ± 1
Muscarine	3	0.42 ± 0.13	1.05 ± 0.19	95 ± 5
Arecoline	3	0.69 ± 0.27	0.84 ± 0.02	100 ± 1
Bethanechol	8	34.4 ± 13.4	0.84 ± 0.09	77 ± 6
McN-A343	4	1.4 ± 0.28	0.86 ± 0.13	68 ± 4
Pilocarpine	4	10.1 ± 2.9	1.04 ± 0.15	52 ± 5
CHO-2 cells				
Oxotremorine-M	27	0.35 ± 0.02	1.11 ± 0.07	100 ± 6
Carbachol	8	2.8 ± 0.76	1.38 ± 0.2	100 ± 0.4
Oxotremorine	8	0.2 ± 0.02	0.83 ± 0.09	97 ± 3
Muscarine	6	5.1 ± 1.7	1.26 ± 0.1	92 ± 4
Arecoline	4	2.02 ± 0.81	0.77 ± 0.11	88 ± 6
Bethanechol	7	19.5 ± 3.4	0.79 ± 0.11	101 ± 3
McN-A343	5	17.5 ± 3.9	0.84 ± 0.24	28 ± 1
Pilocarpine	5	11 ± 3.4	1.28 ± 0.27	43 ± 3

value found by the comparative method with the reference agonists (22.7 μM). The binding dissociation constant of pilocarpine in the striatum appears more "M2-like" than it does in the homogeneous M4 system in N1E-115 cells. Thus, the binding potency of pilocarpine appears to distinguish between the M4 receptors in N1E-115 cells and striatum, whereas the higher potency of oxotremorine at the M2 receptor may help to distinguish this receptor from the M4 receptor.

The relative intrinsic efficacies for the six agonists, in comparison with oxotremorine-M, are shown in Tables 1–3, with a summary by rank ordering shown in Table 5. Additionally, the efficacies were also calculated from the ratio of occupancies (P_1/P_2), using the fitted parameters for the concentration-response curves shown in Table 4 and the K_d values found by the agonist-agonist null method or the method of partial receptor alkylation. In general, these efficacy values were in reasonable agreement with those found using the equations in Experimental Procedures. The most notable exception was for oxotremorine in the striatum. In the comparative method its efficacy was 1.23, but using the ratio of P_1/P_2 a much higher efficacy value (10) was obtained. This higher value results from the fact that the dissociation constant (16.4 μM) used in the calculation was obtained from Furchgott analysis. The method of partial receptor alkylation was used in this case because the calculated efficacy of oxotremorine (1.23) was essentially equal to that of the reference agonist. It is not apparent why the two efficacy measurements differ so greatly for oxotremorine; in other cases in the striatum in which the Furchgott-derived binding dissociation constant was used (for arecoline and muscarine), the efficacy calculations agreed reasonably well.

In general, the efficacies of most of the agonists studied did not differ greatly (i.e., by less than 5-fold) from that of the reference agonist oxotremorine-M, indicating that the M2 and M4 receptors recognize a wide variety of agonist structures fairly similarly. There was a greater range in efficacy values in the M2 system (1.0 to 0.04) than in the M4 systems (1.0 to 0.11 or 0.17). Again, as with the binding dissociation constants, the two M4 systems are not absolutely identical to each other, but they do show certain common differences from the M2 system, particularly for a subset of agonists that were less efficacious in one or more of the receptor systems. In the N1E-115 and striatal systems (see Table 5), the rank order of intrinsic efficacies for these partial agonists was arecoline > bethanechol = McN-A343 ≥ pilocarpine. However, the M2 receptor displayed a different rank order, bethanechol > arecoline > pilocarpine > McN-A343. With respect to the absolute values of relative intrinsic efficacies, McN-A343 was less efficacious and bethanechol was more efficacious at the M2 receptor than these agonists were at either of the M4 receptors. Thus, in considering intrinsic efficacies, it appears that bethanechol and McN-A343 are best at distinguishing the M4 systems from the M2 system.

To display the efficacy results of Tables 1–3 by a graphical means, response versus log(occupancy) plots were constructed for oxotremorine-M, arecoline, bethanechol, McN-A343, and pilocarpine for the three systems (Figs. 7–9). In these figures the occupancies of the reference agonist oxotremorine-M in all three systems, and of arecoline in the striatum, were calculated from the K_d values obtained from the partial receptor occlusion experiments, whereas the occupancies in all other cases were calculated from the binding dissociation constants (K_d values) obtained by the oxotremorine-M/agonist null method. The nearly identical profiles for these agonists in the N1E-115 cells (Fig. 7) and the striatum (Fig. 8), as well as the differing rank order in the M2 system (Fig. 9), are evident. In this type of plot, the lateral displacement of the curves from the reference agonist is a measurement of the relative intrinsic efficacies of the other drugs.

Discussion

For M2 and M4 receptors, null methods were used to obtain the binding dissociation constants and relative intrinsic efficacies for several agonists, compared with the reference agonists oxotremorine-M and/or carbachol. Although the two M4 systems were not identical, they were more similar to each other in certain respects than they were to the M2 system. The rank order of efficacies for four agonists (arecoline, McN-A343, bethanechol, and pilocarpine) for the rat striatal muscarinic receptor-mediated inhibition of cyclic AMP was almost identical to the rank ordering of these agonists in the N1E-115 neuroblastoma clone, but these four agonists ranked in a different order of efficacy in the CHO-2 cells. Bethanechol was more efficacious at M2 receptors and McN-A343 was more efficacious at M4 receptors. Both the M4 systems also differed from the M2 receptor in CHO-2 cells in the binding potency for oxotremorine. The striatal and N1E-115 M4 receptors were different from each other mainly with regard to the binding potency of pilocarpine, indicating that the receptor systems in these preparations are not identical.

The binding dissociation constants for drugs may differ between tissues due to ionic or other (e.g., intracellular concentrations of GTP) assay conditions; however, the same buffer was used for the assays in all three of these systems. Thus, if the pharmacological differences for pilocarpine and oxotremorine at M4 receptors in N1E-115 cells and striatum are due to an assay condition, this condition would likely derive from the tissues themselves. One major difference is that the N1E-115 cell line is nominally a homogeneous population of receptors and cells, whereas in the striatal preparation there are both M2 and M4 receptors present. Further, the striatal muscarinic receptors are localized in a variety of neuronal elements, including terminals modulating the release of excitatory amino acids (cortical-striatal system) and dopamine (nigral-striatal system). These complications may underly some of the differences between the two M4 systems or some of the similarities

TABLE 5

Rank orders of efficacies for agonists in the M4 and M2 receptor systems

If a greater than sign appears to the left of an agonist, then its efficacy is significantly less (larger in value) than that of at least one of the agonists preceding it in the sequence. Between the M4 systems, only the values for arecoline are significantly different.

System	Rank order[a]
N1E-115 cells (M4)	Oxo-M > Oxo > Musc = Are > Beth = McN-A343 = Pilo 1.0 0.8 0.6 0.6 0.3 0.2 0.2
Striatum (M4)	Oxo-M = Oxo = Musc = Are > Beth = McN-A343 ≥ Pilo[b] 1.0 1.2 0.9 1.1 0.4 0.2 0.1
CHO-2 cells (M2)	Oxo-M = Musc = Oxo = Beth > Are > Pilo > McN-A343 1.0 0.9 0.6 0.6 0.3 0.1 0.04

[a] Oxo-M, oxotremorine-M; Oxo, oxotremorine; Musc, muscarine; Are, arecoline; Beth, bethanechol; Pilo, pilocarpine.

[b] The efficacy of pilocarpine in striatum is not significantly different from that of McN-A343 but is significantly different from that of bethanechol ($p < 0.05$).

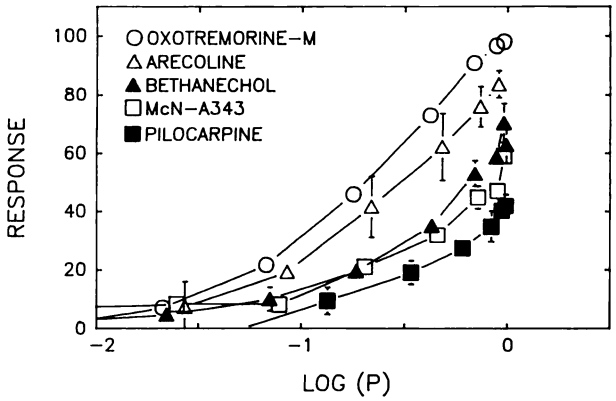

Fig. 7. Composite of relative intrinsic efficacies for eight agonists in the inhibition of cyclic AMP levels in N1E-115 cells. The log (occupancy, *P*) was calculated using the K_d value determined from Furchgott analysis (for the reference agonist oxotremorine-M) or by the plotting method for determining intrinsic efficacy (see Experimental Procedures). Table 1 lists numbers of independent experiments and parameter values obtained from these analyses.

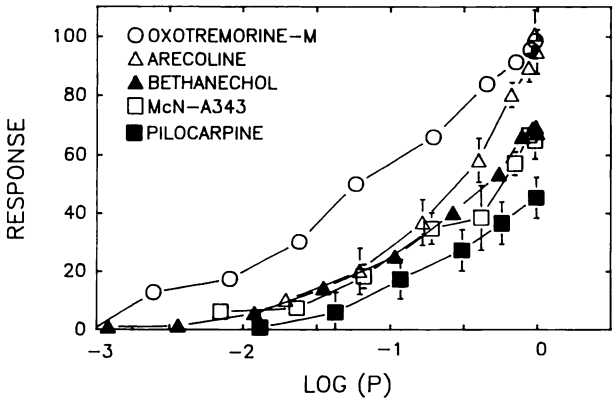

Fig. 8. Composite of relative intrinsic efficacies for eight agonists in the inhibition of cyclic AMP levels in rat striatum. The log (occupancy, *P*) for each agonist was calculated using the K_d value determined from Furchgott analysis (for the reference agonist oxotremorine-M and for arecoline) or by the plotting method for determining intrinsic efficacy (see Experimental Procedures). Table 2 lists numbers of independent experiments and parameter values obtained from these analyses.

between the striatum and CHO-2 cells. It should be noted that there were more differences in agonist efficacies between the two clonal systems studied (M4 in N1E-115 and M2 in CHO-2 cells) than there were between the striatal M4 receptor and the M2 receptor in the CHO-2 cells. Probably, the pharmacological distinctions between M2 and M4 subtypes can be more clearly derived from data for the receptors in these cell lines, which are presumably homogeneous.

It is also possible that tissue variations of GTP-binding protein concentrations could affect the comparison of agonist efficacies. However, one recent study indicated that the concentration of adenylate cyclase rather than the concentration of GTP-binding protein may be the major issue with which to be concerned (21). The fold stimulation by forskolin or PGE_1 of cyclic AMP levels over basal levels was different in the three systems we studied (averages, 10-fold in N1E-115; 6-fold in striatum; and 23-fold in CHO-2 cells), but this may be due to differing amounts of G_s or degrees to which the precursor ATP pool is labeled, rather than differing amounts of adenylate cyclase. Further, the degrees to which cyclic AMP levels were

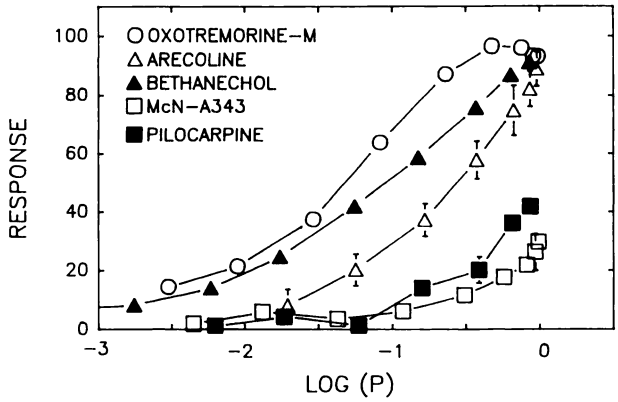

Fig. 9. Composite of relative intrinsic efficacies for eight agonists in the inhibition of cyclic AMP levels in CHO-2 cells. The log (occupancy, *P*) was calculated using the K_d value determined from Furchgott analysis (for the reference agonist oxotremorine-M) or by the plotting method for determining intrinsic efficacy (see Experimental Procedures). Table 3 lists numbers of independent experiments and parameter values obtained from these analyses.

maximally inhibited (generally 35–60% of the forskolin- or PGE_1-elicited level) were fairly uniform between the three tissues.

Our conclusion that the M2 and striatal systems differ with regard to the recognition of agonists is similar to that reached by Keen and Nahorski (6). However, our methods and results differ from theirs in some respects. In our study we examined more agonists and used a null method for determining relative intrinsic efficacies, whereas they used a radioligand binding assay to deduce the K_d value for the agonists for the active conformation (6). In that study the rat striatum was compared with the rat heart, an M2 system, and differing efficacy rank orders for three agonists were obtained. In contrast, we used the method of partial receptor alkylation to obtain the binding dissociation constant for a reference agonist and with this value and the null method calculated the intrinsic efficacies and binding dissociation constants for other agonists. In our minds, at least, it appears reasonable to use Furchgott's method (19) to obtain the binding dissociation constant. This is because we believe that the value obtained should reflect the active conformation of the agonist-receptor-effector conformation in the same system, buffers, etc., in which the agonist concentration-response curves were obtained for application of the null method. We recognize that in the literature there is a controversy regarding the appropriateness of using the method of partial receptor alkylation (22); however, studies by some investigators still support the usefulness of obtaining the agonist K_d value with the classical method (23). What appears to limit more refined modeling of these "G-coupled" systems is the lack of knowledge regarding the local concentrations and thermodynamic/kinetic constants of interaction for all the elements (neurotransmitter, receptor, GTP-binding protein(s), adenylate cyclase, GTP, Mg^{2+}, etc.) involved in transducing the signal from neurotransmitter binding to second messenger formation, which results in an inability to account for the degree to which the receptor is distributed between different states. At the moment, therefore, it seems that the method of partial receptor alkylation is at least an heuristically appealing means of estimating the constant of interaction of agonist and receptor in kinetically very complicated systems.

Our findings confirm that the rat striatal muscarinic system

inhibiting cyclic AMP involves a "noncardiac" muscarinic receptor, a conclusion we (1, 16) and others (3, 4) previously reached with studies of antagonist selectivities. The facts that the M4 binding site composes a substantial fraction of muscarinic receptor binding in striatum (5), that the M4 mRNA molecule is prevalent in this region (2), and that both antagonist and agonist profiles are similar to that of the M4 in N1E-115 cells (16) (present study) lead us to believe that this noncardiac receptor is the M4 gene product. However, as a caveat, some of the disparities between the M4 receptors in N1E-115 cells and striatum may be due to the fact either that these M4 receptors differ in certain subtle ways or that the striatal assay system actually contains a mixture of the M4 and M2 receptors. The combination of approaches used in the present study, nevertheless, helps to establish the degree to which muscarinic agonists can be used to compare or identify functionally coupled muscarinic gene products in the central nervous system. It appears that bethanechol and McN-A343 are the most useful agonists for distinguishing the M2 and M4 muscarinic receptor subtypes.

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