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# Muscarinic receptor occupation and receptor activation in the guinea-pig ileum by some acetamides related to oxotremorine

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**1** The dissociation constants ($K_D$ values) and relative efficacies of seven acetamide analogues of oxotremorine, including two enantiomeric pairs, at muscarinic receptors in the guinea-pig isolated ileum were determined. The method used involved analysis of dose-response data before and after fractional inactivation of receptors with propylbenzilylcholine mustard.

**2** All of the compounds studied had lower affinities than oxotremorine, but some had substantially higher relative efficacies.

**3** Replacement of the pyrrolidine ring of N-methyl-N-(4-pyrrolidino-2-butynyl)acetamide(I), the parent compound in the series, by a dimethylamino or a trimethylammonium group decreased the affinity 32 and 4.5 fold, respectively, whereas the relative efficacy increased 5.7–8.3 times.

**4** There was no correlation between relative efficacies and affinities of the compounds. The structural requirements for high affinity and high efficacy appeared to be quite different.

## Introduction

The pharmacological activity of muscarinic agonists, as measured for example by contractile responses in the guinea-pig isolated ileum, is a function of both the affinity for the receptor and the efficacy in activating the receptor subsequent to formation of the drug-receptor complex (Stephenson, 1956). The structural requirements for high affinity at muscarinic receptors are fairly well known from numerous studies of antagonists (Inch & Brimblecombe, 1974). In contrast, the structural features of muscarinic agonists that cause them to activate the receptor are less readily assessed and are virtually unknown. Separation of the pharmacological activity of agonists into affinity and efficacy components will permit an assessment of the structural demands on agonists with respect to both affinity and efficacy.

Recently, I showed that a modification of the method of Furchgott (Furchgott, 1966; Furchgott & Bursztyn, 1967) provided a good estimate of dissociation constants and relative efficacies at ileal muscarinic receptors of some partial agonists related to the potent muscarinic agonist, oxotremorine (N-(4-pyrrolidino-2-butynyl)-2-pyrrolidone) (Ringdahl, 1984). I also showed that among the analogues studied, the structural requirements for high affinity and high efficacy were quite different, indicating that these two parameters of drug action are independent of one another. In the present paper, I have applied the same method to separate the muscarinic potency of some acetamide analogues (I – V, Figure 1) of oxotremorine in the guinea-pig isolated ileum into affinity and efficacy components. The results will be discussed in terms of structure-activity relationships for muscarinic receptor occupation and receptor activation.

## Methods

Guinea-pigs (male, English short hair, 350–400 g) were killed by a blow to the head and bled. Segments of the ileum (about 2 cm long) were removed and suspended in a 10 ml organ bath containing Tyrode solution at 37°C and continuously gassed with 5% $CO_2$ plus 95% $O_2$. The Tyrode solution had the following composition (mM): NaCl 137, $NaHCO_3$ 12, glucose 5.0, KCl 2.7, $MgSO_4$ 1.0, $NaH_2PO_4$ 0.4, $CaCl_2$ 1.8 (pH 7.4). Hexamethonium ($3 \times 10^{-4}$ M) was always included in the Tyrode solution. Contractions were recorded isotonically at 1 g

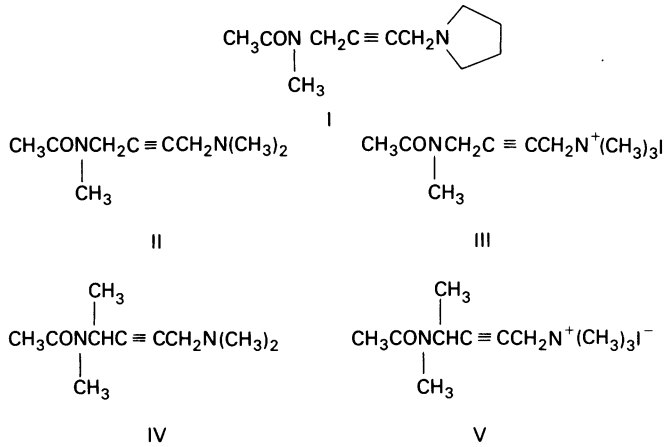


**Figure 1** Chemical structures of compounds I–V.

tension, using an electromechanical displacement transducer and a potentiometric recorder. The ileal strips were allowed to equilibrate for at least 1 h before drug addition. Dose-response curves were constructed by the cumulative dose-response technique (Van Rossum, 1963).

*Determination of dissociation constants ($K_D$) and relative efficacies*

Dissociation constants and relative efficacies of oxotremorine, compounds I–III and of the enantiomers of compounds IV and V at muscarinic receptors of the guinea-pig isolated ileum were determined according to a modification of the method of Furchgott & Bursztyn (1967). After the construction of the control dose-response curves, from which the $ED_{50}$ values were obtained, the preparation was treated with propylbenzilylcholine mustard (PrBCM) (Young *et al.*, 1972; Burgen *et al.*, 1974) to inactivate irreversibly a fraction of the receptors. The tissue was washed for 30 min and the dose-response curve of the agonist was then obtained in the PrBCM treated tissue. The concentrations of PrBCM used were $2 \times 10^{-8}$–$2 \times 10^{-7}$ M for oxotremorine and compounds I, IV and V and $4$–$7 \times 10^{-6}$ M for compounds II and III. These concentrations applied for 15 min reduced the maximal response to these agonists by 20 to 80%, a reduction suitable for determination of agonist dissociation constants (Besse & Furchgott, 1976). In all of these experiments, PrBCM was cyclized at a concentration of $2 \times 10^{-4}$ M in 10 mM phosphate buffer (pH 7.4) for 45 min at room temperature and then kept on ice until required.

Several equieffective concentrations of each stimulant drug before [A] and after [A′] treatment with PrBCM were determined graphically; 1/[A] was plotted versus 1/[A′] and a straight line was fitted to the data by linear regression analysis. From the slope and the intercept on the ordinate, the dissociation constant ($K_D$) of the agonist-receptor complex and the fraction (q) of receptors still active were calculated (Furchgott & Bursztyn, 1967).

The efficacies of compounds I–III and of the enantiomers of compounds IV and V relative to that of oxotremorine were determined from the respective $K_D$ and $ED_{50}$ values, as previously described (Ringdahl & Jenden, 1983).

Fractional occupation of receptors by each stimulant drug at each concentration [A] was obtained from the relationship

$$\frac{[RA]}{[R_T]} = \frac{[A]}{K_D + [A]} \qquad (1)$$

where [RA] is the concentration of receptor-agonist complex and $[R_T]$ is the total receptor concentration (Furchgott & Bursztyn, 1967; Besse & Furchgott, 1976).

This series of oxotremorine analogues was evaluated in parallel with another group of compounds, including oxotremorine and compound I, previously described by Ringdahl (1984). Therefore, the values for oxotremorine and compound I presented here are the same as those previously found.

*Drugs*

Compounds I (Svensson *et al.*, 1978), II and III (Resul *et al.*, 1983), R–IV, S–IV, R–V and S–V (Dahlbom *et al.*, 1982) and oxotremorine sesquioxalate (Bebbington & Shakeshaft, 1965) were prepared as previously described. Other drugs and their sources were the following: hexamethonium chloride (K & K Laboratories, Plainview, NY, U.S.A.) and propylbenzilylcholine mustard (PrBCM) (generous

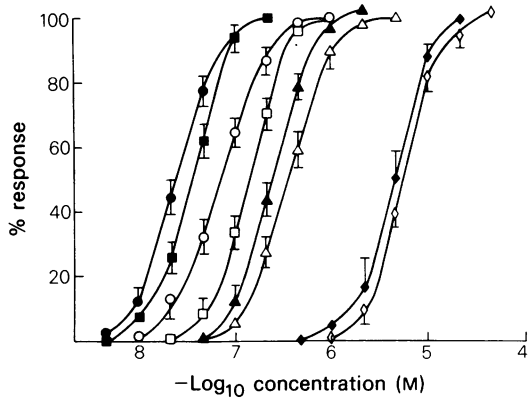


**Figure 2** Dose-response curves in the guinea-pig isolated ileum of oxotremorine (●), compounds III (■), I (○), R–V (□), R–IV (▲), II (△), S–V (◆) and S–IV (◇). Responses are expressed as a percentage of the maximum contraction elicited by oxotremorine. Each point is the mean response and the vertical bars show s.e.mean. The number of experiments is given in Table 1.

gift of Dr N.J.M. Birdsall, National Institute for Medical Research, Mill Hill, London).

## Results

### *Dose-response relationships*

Dose-response curves for oxotremorine, compounds I–III and for the enantiomers of compounds IV and V in the guinea-pig isolated ileum are shown in Figure 2. The dose-response curves of compounds II and III as well as of the enantiomers of compounds IV and V were similar in shape and maximum response to those of oxotremorine and compound I indicating full agonist activity (Ringdahl, 1984). The $ED_{50}$ values, summarized in Table 1, were in good agreement with those previously found (Dahlbom *et al.*, 1982; Ringdahl *et al.*, 1982; Resul *et al.*, 1983).

### *Dissociation constants and relative affinities of compounds I–V*

The results of a typical experiment for the determination of the dissociation constant ($K_D$) of compound III are shown in Figure 3. The $K_D$ values and relative affinities at muscarinic receptors in the guinea-pig isolated ileum of the compounds studied are summarized in Table 2. As previously noted (Ringdahl, 1984), opening of the lactam ring of oxotremorine to give compound I reduced the affinity about 3 fold. Compound I still had the highest affinity of the acetamides studied. Replacement of the pyrrolidine ring of compound I by dimethylamine decreased the affinity 32 fold (compound II), whereas a trimethylammonium group reduced the affinity only 4.5 fold (compound III). Introduction of a methyl group at position 1 of the butynyl chain of compounds II and III produces chiral compounds (IV and V). The R-enantiomer of compound IV had an affinity 11 times higher than the corresponding unsubstituted derivative (II), whereas S–IV had only a slightly lower affinity than compound II. The methyl group of R–V, however, increased the affinity only 1.4 fold as compared to compound III. S–V had an affinity 6 fold lower than compound III.

There was a correlation ($n=8$; $r=0.85$; $P=0.0072$) between the negative logarithms of the $K_D$ (pK) and $ED_{50}$ ($pD_2$) values of oxotremorine and compounds I–V. The regression line was described by: $pK = 0.75 \times pD_2 + 0.02$.

**Table 1** Parameters characterizing the muscarinic activity of some oxotremorine analogues in the guinea-pig isolated ileum

| *Compound* | n | *$ED_{50}$* (M) | *$E_{max}$** | *Relative potency*† |
|---|---|---|---|---|
| I | 6 | $6.94 \pm 0.23 \times 10^{-8}$ | $1.01 \pm 0.01$ | 0.36 |
| II | 6 | $3.85 \pm 0.28 \times 10^{-7}$ | $1.01 \pm 0.02$ | 0.064 |
| III | 5 | $3.69 \pm 0.13 \times 10^{-8}$ | $0.99 \pm 0.01$ | 0.67 |
| R–IV | 5 | $2.50 \pm 0.11 \times 10^{-7}$ | $1.04 \pm 0.01$ | 0.10 |
| S–IV | 5 | $5.31 \pm 0.36 \times 10^{-6}$ | $1.03 \pm 0.02$ | 0.0047 |
| R–V | 6 | $1.39 \pm 0.04 \times 10^{-7}$ | $0.98 \pm 0.03$ | 0.18 |
| S–V | 6 | $4.54 \pm 0.17 \times 10^{-6}$ | $0.99 \pm 0.02$ | 0.0055 |
| Oxotremorine | 10 | $2.48 \pm 0.18 \times 10^{-8}$ | 1.00 | 1.00 |

$ED_{50}$ and $E_{max}$ values are the means ± s.e.mean.
* Maximum response relative to the maximum for oxotremorine which equals 1.00.
† Calculated by dividing the $ED_{50}$ of oxotremorine by the $ED_{50}$ of each compound.

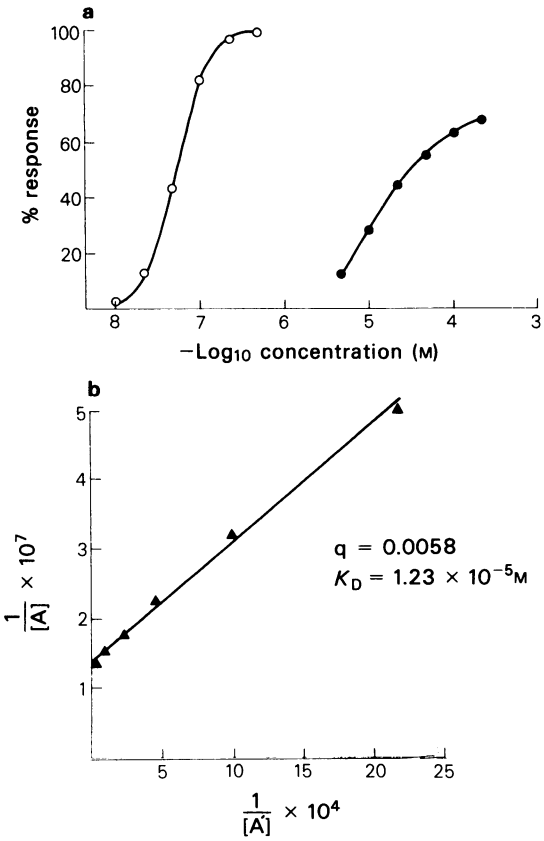


**Figure 3** Dose-response curves for compound III in the guinea-pig isolated ileum (a) and double reciprocal plot of A versus A′ (b). (a) Control dose-response curve (o) and dose-response curve after treatment with $5 \times 10^{-6}$ M PrBCM for 15 min (●). (b) Values for A and A′ were obtained from the control dose-response curve and the plotted points after treatment with PrBCM (a).

*Relative efficacies of compounds I–V*

The relative efficacies of compounds I–V at muscarinic receptors in the guinea-pig isolated ileum varied largely (within a range of 19 fold) (Table 2). As previously shown (Ringdahl, 1984), the efficacy of compound I was not significantly different from that of oxotremorine. Substitution of a dimethylamino or a trimethylammonium group for pyrrolidine in compound I dramatically increased efficacy (compounds II and III). Compound III is the most efficacious oxotremorine analogue found so far. It had greater relative efficacy than carbachol (Ringdahl, 1984) which is regarded as a highly efficacious muscarinic agonist (Birdsall *et al.*, 1978). The methyl substituted analogues IV and V had substantially lower efficacies than the corresponding unsubstituted ones (II and III). The enantiomers of compound IV showed only a small difference in efficacy, whereas R–V had a 3.8 fold higher relative efficacy than S–V.

There was no significant correlation between $pD_2$ values and the relative efficacies ($n = 8$; $r = 0.36$; $P = 0.38$) or between pK values and the relative efficacies ($n = 8$; $r = -0.16$; $P = 0.71$) of the compounds studied.

The relationship between pharmacological response and fractional occupation of receptors in the guinea-pig ileum for compounds I, III and S–V is illustrated graphically in Figure 4. Percentage receptor occupation required for half-maximal response was calculated for each compound from equation (1) in Methods. The results are summarized in Table 2. S–V, the least efficacious compound among the analogues studied, must occupy 7% of the receptors to give one-half of the maximal response, whereas compound III requires only 0.36% receptor occupation.

**Table 2** Dissociation constants and relative efficacies of some oxotremorine analogues at muscarinic receptors in the guinea-pig isolated ileum

| *Compound* | n | $K_D$ (M) | *Relative affinity** | *Relative efficacy* | *% occupancy for 50% response†* |
|---|---|---|---|---|---|
| I | 6 | $2.18 \pm 0.31 \times 10^{-6}$ | 0.31 | 1.14 ± 0.13 | 3.1 |
| II | 6 | $6.99 \pm 1.24 \times 10^{-5}$ | 0.0097 | 6.46 ± 1.14 | 0.55 |
| III | 5 | $1.01 \pm 0.15 \times 10^{-5}$ | 0.067 | 9.50 ± 1.26 | 0.36 |
| R–IV | 5 | $6.40 \pm 0.79 \times 10^{-6}$ | 0.11 | 0.96 ± 0.09 | 3.8 |
| S–IV | 5 | $1.07 \pm 0.15 \times 10^{-4}$ | 0.0063 | 0.74 ± 0.10 | 4.7 |
| R–V | 6 | $7.23 \pm 1.46 \times 10^{-6}$ | 0.094 | 1.89 ± 0.37 | 1.9 |
| S–V | 6 | $6.01 \pm 1.02 \times 10^{-5}$ | 0.011 | 0.50 ± 0.08 | 7.0 |
| Oxotremorine | 6 | $6.79 \pm 1.86 \times 10^{-7}$ | 1.00 | 1.00 | 3.5 |

$K_D$ values and relative efficacies are the means ± s.e.mean.

* Calculated by dividing the $K_D$ of oxotremorine by the $K_D$ of each compound.

† Calculated from equation 1 in Methods as $100 \times \frac{[RA]}{[R_T]}$.

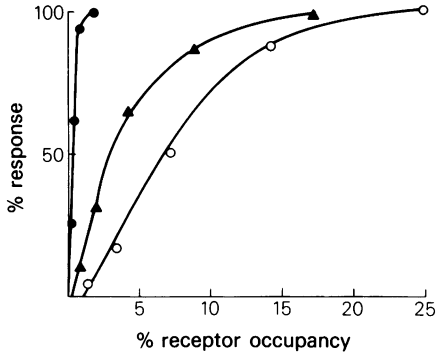


**Figure 4** % response of compounds I (▲), III (●) and S–V (○) as a function of % receptor occupancy. Receptor occupancy at each concentration was calculated from equation 1 in Methods employing the $K_D$ values listed in Table 2.

## Discussion

All of the compounds studied behaved like 'full' agonists (Figure 2). Yet the efficacy of the most efficacious compound was 19 times greater than that of the least efficacious (Table 2), indicating that the amount of spare receptors differed greatly among the compounds. As a consequence, the $ED_{50}$ values for contraction are poor estimates of the affinities of these analogues for the receptor. For example, the estimated $K_D$ value of compound III was 274 times greater than the $ED_{50}$ value measured before treatment of the ileum with PrBCM. The correlation observed between $ED_{50}$ and $K_D$ values thus deviated from a 1:1 relationship and merely indicates that an agonist that is potent at eliciting contractions also has a high affinity for the receptor.

Oxotremorine, having a pK value of 7.91, is mostly protonated at physiological pH and its muscarinic activity is associated with the protonated form (Hanin *et al.*, 1966). The base strengths of the other tertiary amines (compounds I, II and IV) included in this study are not sufficiently different from that of oxotremorine to affect to any great extent a comparison of their pharmacological activities (Resul *et al.*, 1983).

Compound I, first described by Bebbington *et al.* (1966), may be regarded as the parent compound in a large series of muscarinic and antimuscarinic carboxamides structurally related to oxotremorine (Svensson *et al.*, 1978; Dahlbom *et al.*, 1982; Resul *et al.*, 1982; 1983; Ringdahl *et al.*, 1982). Like oxotremorine, it readily penetrates into the brain after systemic administration and causes pronounced stimulation of central muscarinic receptors (Bebbington *et al.*, 1966; Svensson *et al.*, 1978; Resul *et al.*, 1983). Replacement of the pyrrolidine ring of compound I by a dimethylamino (compound II) or a trimethylammonium group (compound III) was accompanied by a marked decrease in affinity and increase in relative efficacy at ileal muscarinic receptors. From the relative affinities and efficacies of compounds I–III (Table 2), it appears that a pyrrolidine ring imposes high affinity and low efficacy on these acetamides (compound I), whereas the presence of a dimethylamino group leads to low affinity and high efficacy (compound II). Introduction of a trimethylammonium group gives rise to a molecule with exceptionally high efficacy which also maintains relatively high affinity (compound III). As a result of its lower relative efficacy, compound I must occupy a substantially greater fraction of the receptor pool to produce the same pharmacological response as compounds II and III (Figure 3). Compound I may therefore be regarded as a partial agonist with respect to compounds II and III.

Addition of a methyl group at position 1 of the butynyl chain of compound I yields a partial agonist (Dahlbom *et al.*, 1982; Resul *et al.*, 1982; Ringdahl, 1984). Analogous structural modifications of compounds II and III gave rise to agonists (compounds IV and V) capable of causing a maximal response in the guinea-pig ileum. However, the enantiomers of compounds IV and V had considerably lower relative efficacies than the corresponding unsubstituted derivatives. The methyl group in the butynyl chain of R–IV increased the affinity, relative to compound II, by about the same amount (11 fold) as the corresponding methyl substitution in compound I and in oxotremorine (Ringdahl, 1984). This observation suggests that the methyl group of R–IV provides an additional point for attachment to the receptor. Since the relative efficacies of R–IV and S–IV differed very little, the 21 fold potency difference between the enantiomers of compound IV may be ascribed almost entirely to differences in affinity. Low stereoselectivity with respect to efficacy was also observed for the enantiomers of two previously studied partial agonists related to oxotremorine (Ringdahl, 1984). The enantiomers of compound V appeared to be exceptional in several ways. Firstly, the methyl group in the butynyl chain of R–V caused only a negligible increase in the affinity as compared to compound III. Secondly, the enantiomers of compound V differed almost 4 fold in relative efficacy and, in contrast to previous observations (Ringdahl, 1984), that enantiomer (R–V) which had the highest efficacy also had the highest affinity. Differences in affinity and in relative efficacy both contribute to the large (33 fold) potency difference between R–V and S–V. These results suggest that there are subtle differences in the binding and receptor activating properties of the tertiary amine IV and the quaternary ammonium compound V.

This study has confirmed previous suggestions that the structural features leading to high affinity are distinct from those leading to high efficacy (Ruffolo *et al.*, 1980; Ringdahl & Jenden, 1983; Ringdahl, 1984). Thus there was no correlation between the relative efficacies and affinities of the compounds studied. While many of the oxotremorine analogues investigated before have higher affinities than oxotremorine, none has been shown to have a significantly greater relative efficacy. Only with the present study have compounds emerged that are more efficacious than oxotremorine. This increase in efficacy was accomplished by chemical modification of the tertiary amino group of compound I. A more extensive series of analogues of oxotremorine modified in the amino group is now the subject of an analysis similar to the one described in the present communication.

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