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INHIBITION OF [^{3}H] DEXETIMIDE BINDING BY A HOMOLOGOUS SERIES OF METHYL-FURTHRETHONIUM ANALOGUES AT THE PERIPHERAL MUSCARINIC RECEPTOR

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Summary. The interaction of a homologous series of methylfurthrethonium analogues, in which there is a gradual transition from full agonists via partial agonists to antagonists,with the peripheral muscarinic receptor was investigated by concentration-dependent inhibition of [^{3}H] Dexetimide binding. The antagonists give normal sigmoid inhibition curves, but those of agonists follow a much flatter course. The results can be explained by assuming the presence of two non-interconverting muscarinic binding sites for which agonists have different and antagonists have identical affinities.

The affinity ratio for the two binding sites decreases from 61 for methylfurthrethonium to 1 for its isopropyl analogue and parallels the decrease in intrinsic activity.

Concentration dependent inhibition of labeled muscarinic antagonists binding by non-labeled antagonists displays normal sigmoid behaviour and can be explained on basis of competition between labeled and non-labeled compounds for a single class of binding sites (1). A prominent feature of inhibition curves of labeled antagonists by non-labeled agonists is a deviation from sigmoid behaviour. The curves are less steep than might be expected on basis of a simple competition. The Hill plots are non-linear and the inclinations in the middle part of the curve strongly deviate from one (2, 3). If it is assumed that there are two independent non-interconverting binding sites for which antagonists have identical or almost identical affinities and for which agonists have different affinities a simple explanation for these experimental findings is offered (4). Although such a two site receptor model accounts for many if not all experimental observations (5, 6), it leaves a few important questions unanswered. Are both binding sites or is only one of them related to the receptor mediating the physiological response and if it is only one, is it the low or the high affinity binding site? It is difficult to comprehend that such structurally diversified antagonists at muscarinic receptors like atropine, quinuclidinyl benzilate, dexetimide and others all bind with the same high affinity to the two postulated sites. Often there are large structural differences between agonists on one hand and antagonists on the other and it is not clear, whether discriminative behaviour towards the two binding sites is confined to one structural class of compounds or whether it is inherent

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to the nature of agonism itself or for that matter to intrinsic acitivity. The study of a homologous series of compounds in which there is a gradual transition from agonist to antagonist such that the compounds can still be seen as belonging to one structural class might bring further enlightenment in this matter.

We wish to report on the inhibition of [^{3}H]Dexetimide binding by a homologous series of methylfurthrethonium analogues.

MATERIALS AND METHODS

[^{3}H]Dexetimide (spec.act. 420 mCi/mmol), unlabeled Dexetimide and (-)-benzetimide were gifts from Janssen Pharmaceutica, Beerse, Belgium. Alkylated methylfurthrethonium analogues were synthesized according to known procedures. Other reagents were obtained from commercial sources.

Bovine tracheal smooth muscle was processed and homogenized with carborundum as described previously (7). The crude membrane pellets were washed once with Krebs-Henseleit by Potter-homogenization and the suspension obtained was centrifuged at 100,000 g for 45 minutes. The resulting pellet was resuspended in Krebs-Henseleit to give a tissue concentration of 0.50 g/ml.

The method used for the assay of particle bound radioactivity was the rapid centrifugation of membrane fragments using a microfuge (8). Assay tubes received 1.00 ml membrane suspension, 0.100 ml of an [^{3}H]Dexetimide solution to give a free concentration of approximately 10^{-8} M corresponding to 95% receptor saturation and 0.100 ml of either Krebs-Henseleit or a solution of unlabeled drug in Krebs-Henseleit to give the concentrations depicted in figure 1. The incubation volume was 1.20 ml. Assays were done in 1.5 ml stoppered polypropylene tubes. The tubes were rotated at 1 rpm for 20 minutes. The membrane fragments were pelleted by centrifugation at 25,000 g for 15 minutes in a Christ Heraeus Hämofuge-912. The free concentration of radioactively labeled ligand was measured by mixing 0.50 ml of supernatant with 10 ml of Instagel (Packard) and counting. Radioactivity in the pellet was measured by cutting the tips of the centrifuge tubes, dissolving in 1 ml Soluene (Packard) and mixing with 15 ml of Instagel/1 N HCl (9:1). The samples were counted in a Packard Liquid Scintillation Counter Model 3380 with standard deviations of 1% or less. Free and bound concentrations of labeled ligand were calculated from the radioactivities measured. Free concentrations of unlabeled ligands were calculated from the standard solutions added and were assumed not to differ appreciably before and after equilibrium had been established.

ANALYSIS OF INHIBITION CURVES

Binding of a ligand A to tissue fragments can be described as occurring to a finite number of binding sites with different affinities and capacities for the drug bound. When inhibition of binding by a competing ligand B is also considered, the concentration of drug A bound as a function of the free ligand concentrations A and B can be expressed as

$$[A]_{bound} = \sum_{i=1}^{n} \frac{r_i}{1 + \frac{K_{A_i}}{[A]}\left(1 + \frac{[B]}{K_{B_i}}\right)} \qquad (1)$$

in which r_i is the concentration of sites of the i^{th} class of binding sites and K_{A_i} and K_{B_i} are the dissociation equilibrium constants of drugs A and

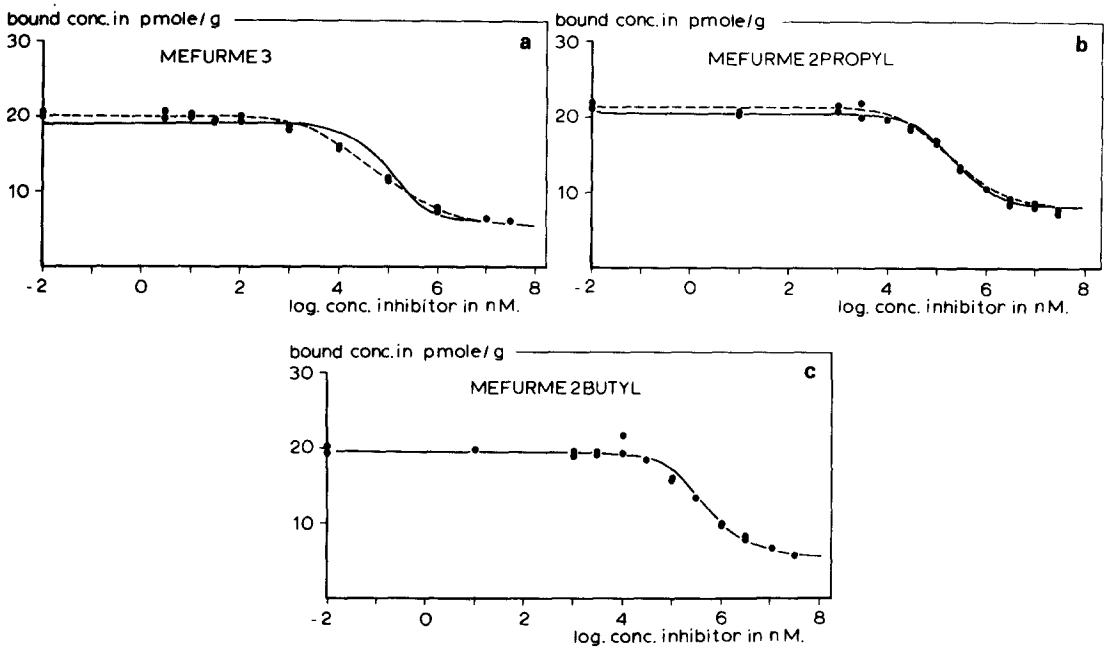


Fig. 1 Inhibition of [^{3}H]Dexetimide binding by methylfurthrethonium (1a), its propyl analogue (1b) and its butyl analogue (1c). Solid curves: normal competitive behaviour. Best fit to experimental data with model 1 (one binding site; see methods). Dashed curves: anomalous competitive behaviour. Best fit to experimental data with model 2 (two binding sites; see methods).

B for the i^{th} class. We have analyzed our inhibition curves on basis of the following models.

Model 1	one specific binding site	(n = 1 equation (1))
Model 2	two specific binding sites	(n = 2 equation (1))
Model 3	two specific binding sites with a fixed capacity ratio	(n = 2 equation (1)) (r_1/r_2 = constant)

The parameters of the theoretical binding models applied were fitted to the experimental data with a computerized curve fitting procedure. The unspecific binding is determined as the residual binding in the presence of a 1000-fold excess of unlabeled drug A. Bound concentrations are calculated by subtracting unspecific binding from total binding. In models 2 and 3 it is assumed that drug A has identical affinities for the two specific binding sites, hence identical values for K_A for the first and the second binding site are taken. The free concentration of the ligand to be displaced is kept as constant as possible at approximately 10^{-8} M.

In comparing the models preference is given to model 2 or 3 over model 1 when a significant diminution in the residual sum of squares is obtained. Significance is assessed by means of the F-test (9).

RESULTS AND DISCUSSION

The inhibition of [^{3}H]Dexetimide binding by a homologous series of methylfurthrethonium analogues reveals normal competitive behaviour for the antagonist amongst them (e.g. the butyl analogue in fig. 1c). The

agonists (e.g. the methyl analogue in fig. 1a) and to a lesser extent the partial agonists (e.g. the propyl analogue in fig. 1b) are anomalous in the sense that inhibition curves are less steep than may be expected on basis of competition for a single binding site and that Hill plots are non-linear. The anomality in the inhibition curves of the agonists can be quantitated by analyzing the data in terms of a two binding site model. Deviation from normal binding behaviour can then be expressed as the ratio of the dissociation constants for the two sites. In view of the definitions of a two site model, a variation in the ratio of the binding capacities of the preexisting sites is contradictory and a correct approach would be to analyze the binding data of the different congeners of the series by a two binding site model with a fixed ratio of the binding capacities (model 3). The best value for this ratio appears to be one as estimated from the inhibition of [^{3}H]Dexetimide binding by methylfurthrethonium. For reason of comparison the complete series of analogues is analyzed according to model 3. The result of the analysis is summarized in table 1, together with values for the F-test in which the significance of the models applied is checked. The Hill coefficients, which can be seen as a semiquantitative measure for deviation from simple mass action behaviour, are included in table 2. The F-test confirms that in case of agonists and to a lesser extent in case of partial agonists, a two binding site model describes the results significantly better than a one binding site model. Comparison of $F_{2,n-4}$ with $F_{1,n-3}$ (table 1) makes it clear that preference should be given to the two binding site model with a fixed capacity ratio of one.

The binding phenomena as such can easily be explained by postulating two muscarinic binding sites for which antagonists have identical and agonists have different affinities. These results indicate that even within a homologous series of compounds agonists show a deviant binding behaviour as compared to antagonists. We feel that anomalous binding behaviour is not confined to a particular structural class of compounds but is in one way or another connected with the very nature of agonism itself (10). Having the binding phenomena explained by supposing two muscarinic binding sites, the question remains which of the two is mediating the biological response and what is the physiological role of the other one? Comparison of the pharmacological parameters and the binding parameters obtained with model 3 (table 2) shows a correlation of the pD_2/pA_2 values and $-\log K_1$ ($r = .82$; $p < .1$).

The ratio K_2/K_1 parallels the decline in intrinsic activity along the series of compounds; agonists have a high K_2/K_1-ratio, partial agonists have an intermediate K_2/K_1-ratio and antagonists have a value for K_2/K_1

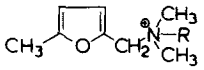
Table 1 Analysis of the inhibitors of [^{3}H]Dexetimide binding by methylfurthrethonium derivatives

Compound		Binding parameters according Model 3			F^b-values	
		K_1 (10^{-6}.M)	K_2 (10^{-6}.M)	$r_{t1} = r_{t2}$ (10^{-12} mol/g)	$F_{2,n-4}$	$F_{1,n-3}$
$MeFurMe_3$	I (22)[a]	0.32 (0.07)	18.1 (2.0)	7.4 (0.1)	35**	75**[c]
	II (30)	0.22 (0.06)	15.8 (1.9)	6.9 (0.2)	34**	56**
$MeFurMe_2Et$	I (22)	0.32 (0.13)	10.1 (1.0)	6.6 (0.3)	36**	22**
	II (32)	0.37 (0.17)	5.5 (1.2)	4.2 (0.2)	5.2*	6.7*
$MeFurMe_2Pr$	I (23)	2.9 (0.8)	20.4 (3.0)	6.7 (0.1)	5.4*	6.5*
	II (34)	3.6 (0.8)	23.6 (3.3)	8.7 (0.2	5.6*	8.3*
$MeFurMe_2Allyl$	I (23)	2.0 (0.7)	25.3 (4.6)	8.6 (0.2)	4.8*	10**
	II (24)	1.9 (0.4)	17.9 (1.7)	7.9 (0.1)	12**	25**
$MeFurMe_2iPr$	(24)	$K_1 = K_2 = 8.4$ (0.8)		8.4 (0.3)	NS	NS
$MeFurMe_2Bu$	(24)	$K_1 = K_2 = 13.8$ (1.1)		7.1 (0.1)	NS	NS

Table 1 K_1 and K_2 are the dissociation constants for the high and low affinity binding sites respectively. r_{t1} and r_{t2} are the corresponding binding capacities. Values in parenthesis indicate standard errors. In the model applied (see methods) it is assumed that $r_{t1} = r_{t2}$. The dissociation constant of Dexetimide has been determined earlier to be 0.4 nM (7) and is assumed to be equal for both binding sites. I, II refer to duplicate experiments. a) number of data points; b) $F_{2,n-4}$ compares model 2 with model 1 for significance; $F_{1,n-3}$ compares model 3 with model 1; c) * = $p < 0.05$, ** = $p < 0.01$ NS = not significant.

of unity. All this points to the high affinity binding site as the one directly involved in mediating the biological response. Birdsall et al. (11) argue that such a relationship must be spurious because pD_2-values do not reflect true affinities as a result of the existence of spare receptors and that affinity constants derived from contractile responses in smooth muscle after spare receptors have been removed with an irreversibly reacting antagonist are better correlated with the affinity constants of the low affinity binding site. In our view it is doubtful whether the concept of spare receptors can provide arguments in favour of or against one of the two binding sites being involved in mediating the biological response. The concept of spare receptors finds its basis in the assumption of a simple bimolecular interaction between drug and receptor. In the more complex situation of the muscarinic receptor as it is apparent from binding experiments the concept of spare receptors requires redefinition especially with regard to irreversibly reacting compounds.

Table 2. Comparison of binding parameters with in vivo drug parameters in a series of methylfurthrethonium analogues.



R	α	pD_2/pA_2	Model 3		
			- log K_1	K_2/K_1	n_H
$-CH_3$	1.0	7.2	6.6	61	0.41
$-CH_2-CH_3$	1.0	6.3	6.5	18	0.58
$-CH_2-CH=CH_2$	-	-	5.7	9.8	0.72
$-CH_2-CH_2-CH_3$	0.9	5.3	5.5	6.8	0.80
$-CH(CH_3)_2$	0	5.8	5.1	1.0	1.0
$-CH_2-CH_2-CH_2-CH_3$	0	4.4	5.2	1.0	1.0

Table 2 K_1 and K_2 are the dissociation constants for the high and low affinity binding sites respectively, n_H = Hill coefficient. pD_2/pA_2-values and intrinsic activity (α) values are taken from van Rossum and Hurkmans (14) and were obtained from contractile responses in isolated rat intestine.

An attractive interpretation of the binding results is offered by receptor models which start from a trimolecular interaction in which besides the drug molecule and receptor molecule a third molecule like an effector molecule (12) or a regulatory protein molecule (13) is involved.

Calculations which will be published elsewhere have shown that inhibition of [^{3}H]Dexetimide binding by methylfurthrethonium and its analogues can be described equally well by such a trimolecular interaction model.

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