A NOVEL APPROACH TO THE QUESTIONS OF ALLOSTERIC PROPERTIES OR A 'RECEPTOR RESERVE' OF DRUG BINDING SITES OF INTESTINAL SMOOTH MUSCLE CELLS*

Dida KUHNEN-CLAUSEN
Institut für Aerobiologie der Fraunhofer-Gesellschaft
D 5949 Grafschaft/Sauerland, GFR

Received 5 November 1973

1. Introduction

The theories of drug–receptor interactions are extensively discussed on the basic assumtption, that potent cell-stimulating agents need to occupy only a small fraction of the total receptor concentration to generate maximal response of the effector cell [1–4]. This assumption implies the presence of a 'receptor reserve' and it founded in attempts to reduce the concentration of particular ligand receptors by means of irreversible blocking compounds. Frequently, the 2-halogenoethylamine Dibenamine has been used, according to the concept [5], that Dibenamine alkylates directly those smooth muscle cell receptors with which the drug itself must combine to produce a response [3, 4, 6]. Recently, this theory has been contradicted [7]. The supposition was made, that alkylating agents produce nonspecific allosteric interactions with multiple binding sites of the rat jejunum.

In studies on the parasympatholytic effects of quaternary pyridines on the isolated guinea pig ileum [8], a treatment with Dibenamine caused changes of the activities of some pyridines. These findings are not consistent with the elimination of a fraction of cholinergic receptors. They are linked to the assumption of allosteric effects as a consequence of the treatment by Dibenamine.

* Some graphs of this contribution have been exposed on the occasion of the Ninth International Congress of Biochemistry, Stockholm 1973.

The dose response curves to cholinergic ligand, acting at the level of muscarinic receptors of intestinal smooth muscle cells, are S-shaped and their Hill coefficients differ clearly from unity [9]. This parallels to the dose response curves of the nicotinic acetylcholine receptor of the electric organ of *Electrophorus electricus* [10–12], and to the kinetics of regulatory enzymes [13, 14]. An analogy of the regulatory properties of cholinoceptive enzymes and receptors has been proposed [9].

In this letter, the actions of cholinergics and cholinolytics on the muscarinic receptor of the isolated longitudinal muscles of the guinea pig ileum are reported and discussed. Attempts were made to protect muscarinic or histaminic receptors against the irreversible blockade by Dibenamine with specific and nonspecific ligands. The phenomena are discussed in regard to the theory of the 'receptor reserve'.

2. Materials and methods

Longitudinal muscle strips, isolated from the guinea pig ileum according to Paton and Rang [15] were suspended in an organ bath at 37°C, containing Tyrode solution of the following composition (mM/litre): NaCl 137; KCl 3.7; $CaCl_2$ 1.8; $MgCl_2$ 1.05; NaH_2PO_4 0.2; $NaHCO_3$ 11.9; glucose 5.5. The bath was gassed with carbogen, the pH was 7.4–7.5. Isotonic contractions were recorded on a kymograph. The load of the lever was 150 mg. Cumulative dose response curves to the cholinergic or histaminic li-

gands were performed as described elsewhere [8].

Acetyl-β-methylcholine bromide (MeCh) and 2-furfuryl-trimethyl-ammonium chloride ($HFurMe_3$) were used for muscarinic, histamine-dihydrochloride (histamine) for histaminic receptor stimulation. The benzylate ester of ethyldimethyl-(2-hydroxymethyl) ammonium chloride (lachesine), 1,1′trimethylene-bis-(4-formylpyridinium bromide) dioxime (TMB-4) and 1,1′oxydimethylene-bis-(4-formyl-pyridinium chloride) dioxime (Toxogonin) served as antagonists. *N,N*-Dibenzyl-2-chlorethylamine hydrochloride (Dibenamine) was the irreversible inhibitor for muscarinic and histaminic receptors. All experiments were performed in the presence of 10^{-5} M hexamethonium in order to avoid ganglionic stimulation. The concentrations of all compounds are indicated as final concentrations.

3. Results

3.1. *Analysis of the action of cholinergic ligands on the muscarinic receptor*

The response of the muscle strip preparation to MeCh were calculated as a fraction of the maximum contraction or as the percentage of the latter. The data were plotted versus the logarithm of the spasmogen's concentrations. From these dose response curves, concentration producing different standard responses (e.g. 5, 10, 25, 50...% maximum response) have been determined. By this procedure it was possible to plot linear dose response curves with intervals smaller than that of the original dose response curves. Thus the characteristics of the represented curves can be better expressed than that of the original values. The data obtained by this method had been used for the calculation of the Hill coefficients.

Fig. 1 shows the response of the muscle strips as a function of increasing concentrations of MeCh. The transformation of the sigmoidal curve by the Hill equation yields two straight lines with an intercept at a concentration of MeCh, generating approximately 20–30% maximum contraction. Two Hill coefficients 'n_H' can be determined at 10 and 50% maximum response.

The same graph shows, that a known muscarinic antagonist, Lachesine, shifts the dose response curve to MeCh to the right, but does not alter significantly

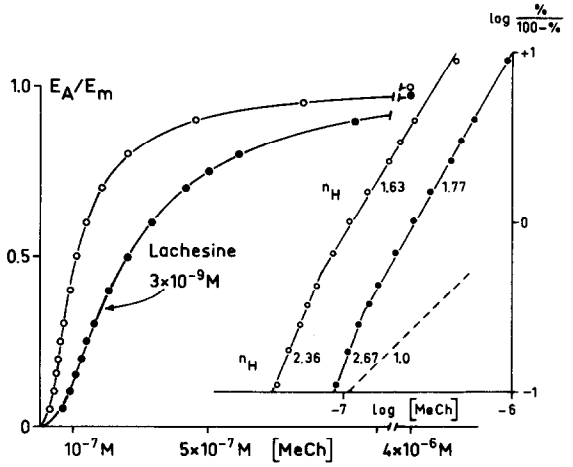


Fig. 1. Cumulative dose response curve to acetyl-β-methylcholine (MeCh) and the effect of the specific antagonist Lachesine on the shape and position of the curve. E_A = dose dependent contraction of the longitudinal muscle strip of the guinea pig ileum as fraction of the maximum effect, E_m, of MeCh. The inset represents the respective Hill plots, with E_A as % of E_m. The Hill coefficients were calculated at the points corresponding to 10 and 50% maximum contraction.

the shape of the curve or the Hill coefficients. The double reciprocal plot of the dose response curves of fig. 1 yields curves, which can be fitted reasonably by a hyperbola.

The quaternary bispyridines Toxogonin and TMB-4 are rather weak antagonists, when compared with Lachesine [8]. In the presence of these compounds, the shapes of the dose response curves to MeCh are more approached to a hyperbola than to the S-form. The Hill coefficients are slightly but significantly decreased.

In the presence of different single doses of MeCh, the dose response curves to Lachesine or Toxogonin are converted from a hyperbola into a S-shaped curve, when the concentration of MeCh exceeds 10^{-6} M. The Hill plots of these curves are reasonably fitted by a straight line with a negative slope (n_H for Lachesine = 2.03, for Toxogonin = 1.76).

Fig. 2 shows dose response curves to MeCh alone and in the presence of one single concentration of another muscarinic receptor stimulator. $HFurMe_3$ shifts to dose response curve to MeCh to the left and converts its shape into a hyperbola. The Hill coeffi-

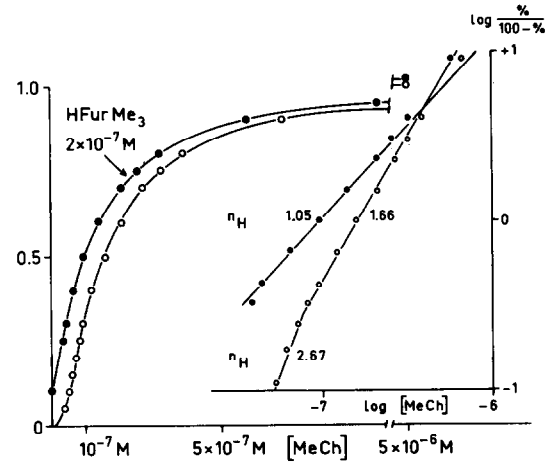


Fig. 2. The effect of the muscarinic agonist furthrethonium ($HFurMe_3$) on the shape and position of the dose response curve to acetyl-β-methylcholine (MeCh). The inset shows the analysis of the curves by the Hill equation. For further explanation see text of fig. 1.

cient of this curve appraches to one. The double reciprocal plot yields a straight line.

3.2. *Protection of cholinergic and histaminic ligand binding sites against the irreversible blockade by Dibenamine*

When the muscle strips are exposed to Dibenamine for 20 min, and nonbound Dibenamine is removed by repeated rinsing, the dose response curves to MeCh or histamine are shifted to the right, and the maximum responses decrease. Recovery does not occur within the experimental period.

In the protection experiments, the organ bath was supplemented with different single concentrations of the respective agonist, or of Lachesine or TMB-4, 2 min before Dibenamine was added. After an incubation for 20 min, the muscle strips were rinsed at least 10 times. Fig. 3a and 4a represent the dose response curves to the agonists after this treatment.

The specific ligand for muscarinic binding sites, MeCh, has only slight protective properties against Dibenamine (fig. 3a). The dose investigated is that needed for maximum response. An increase in the concentration of MeCh does not increase the protective effect. Lachesine prevents the effect of Dibenamide better than MeCh, but here again an increase in the concen-

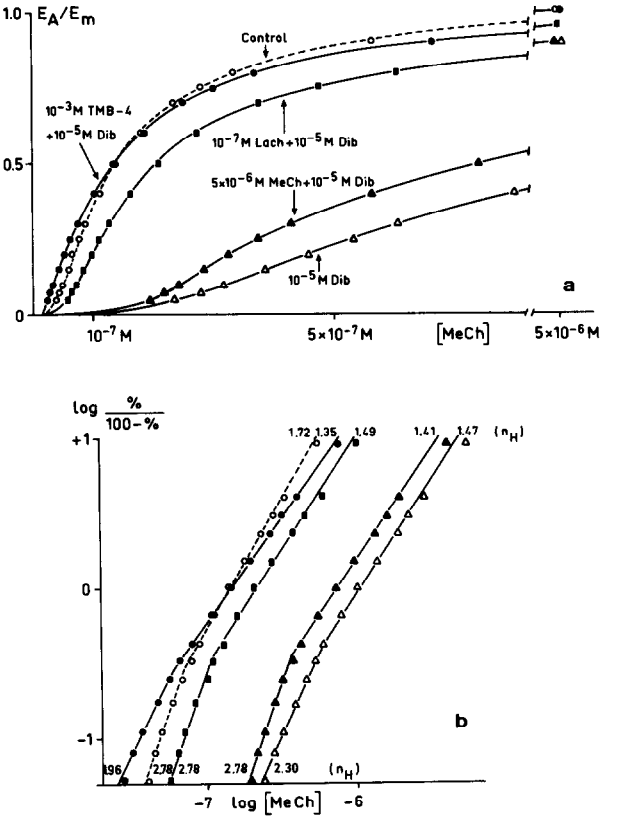
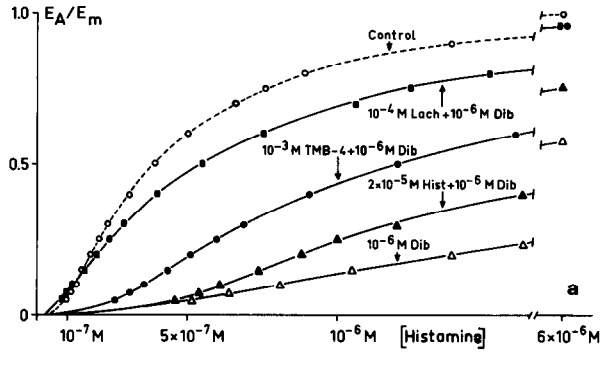


Fig. 3. The effect of the irreversible receptor inactivator Dibenamine (Dib) in the presence and in the absence of acetyl-β-methylcholine (MeCh), Lachesine (Lach) or TMB-4: a) dose response curves to MeCh after the indicates treatment, in the absence of any other compounds (homotropic effects); b) the analysis of the dose response curves to MeCh by the Hill equation. For experimental details see text.

tration over the level, indicated in fig. 3a, does not result in an enhanced protection. The concentration of 10^{-7} M Lachesine has an anticholinergic activity equieffective to 10^{-3} M TMB-4. However, this dose of TMB-4 gives full protection against Dibenamine. Furthermore, the foot of the dose response curve to MeCh is shifted to the left. The shape of the curves approaches to a hyperbola, so that the Hill coefficients of this curve have lower values than those of the control curve and of the curve made after the treatment with Dibenamine alone (fig. 3b).

In order to investigate the specificity of Lachesine and TMB-4 for the protection of muscarinic ligand binding sites, both compounds were used in protec-



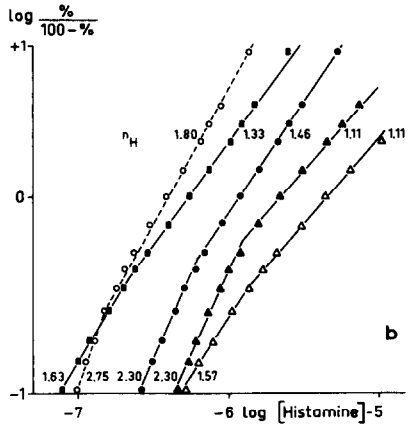


Fig. 4. The effect of the irreversible receptor inactivator Dibenamine (Dib) in the presence and in the absence of histamine (Hist), Lachesine (Lach) or TMB-4: a) dose response curves to the homotropic effects of histamine; b) the analysis of the dose response curves to histamine by the Hill equation. n_H Calculated according to fig. 1.

tion experiments against the alkylation of histaminic binding sites by Dibenamine. Since the recognition sites for histamine are very sensitive to Dibenamine, this agent was applied in a lower concentration than in the experiments with MeCh (fig. 4a). Lachesine and TMB-4 are weak histaminic antagonists. They were investigated in approximately equieffective concentrations. The experimental conditions are the same as mentioned above in regard to fig. 3.

Fig. 4 shows, that the treatment of the muscle strips with Dibenamine shifts doese response curves of histamine to the right, reduces the maximum responses (fig. 4a) and approaches the Hill coefficients to one (fig. 4b). The protective effect of histamine, in a three-fold concentration of that needed for maximum stimulation, is feeble. It is comparable with the protective effect of MeCh (fig. 3). Lachesine and TMB-4 produce protective effects, reversed than on cholinergic binding sites. Now Lachesine is more effective than TMB-4, and the dose response curve to histamine is approached to a hyperbola. The corresponding Hill coefficients (fig. 4b) have values, somewhat higher than those from the dose response curve after the application of Dibenamine alone.

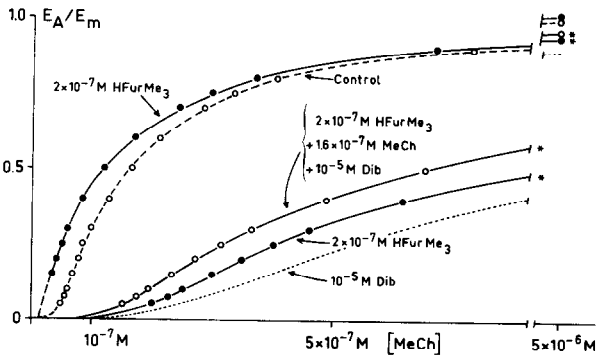


Fig. 5. The effect of furthrethonium ($HFurMe_3$) on the shape and position of the dose response curve to acetyl-β-methylcholine (MeCh) before and after the incubation of the muscle strips with Dibenamine (Dib) in the presence of the combined doses of $HFurMe_3$ and MeCh. Open symbols: dose response curves to MeCh alone (homotropic effects). Closed symbols: dose response curves to MeCh in the presence of $HFurMe_3$ (heterotropic effects). The dotted line represents the inhibitory effect of Dibenamine according to fig. 3. Note that the protective effect against Dibenamine by the combined concentrations of the two muscarinic agonists is more than additive.

In protection experiments at the level of the muscarinic receptor, 2×10^{-7} M $HFurMe_3$ or 1.6×10^{-7} m MeCh (approximately the ED 50) are uneffective against Dibenamine. These concentrations of both receptor activators combined, protect the muscarinic binding site against the inactivation by Dibenamine to the same degree as the 30-fold concentration of MeCh alone (fig. 3a). After this treatment, the simulative effect of 2×10^{-7} M $HFurMe_3$ is lost. In some cases (4 of 7 experiments) the response to MeCh is inhibited instead of being activated, as shown in fig. 5.

4. Discussion

The theory of the 'receptor reserve' [2–4] is based on the assumption, that irreversible inactivation decreases the sensitivity of the effector cell to the agonist only by reducing the concentration of the receptors available for the agonist. Therefore, one common recognition site for the agonist, the irreversible inactivator and for competitive antagonists should be involved in the drug–receptor and drug–drug interaction. The assumption excludes receptor–receptor interaction or cooperativity.

However, from kinetics of regulatory proteins it is known, that S-shaped dose response curves and Hill coefficients above unity indicate cooperativity of ligand interactions [10, 13, 14]. The dose response curves to MeCh or Histamine are S-shaped, too (figs. 1–5). The Hill coefficients differ clearly from one and are often near two. The displacement of the dose response curve to MeCh in the presence of $HFurMe_3$, the conversion of its shape into a hyperbola and the decrease of the Hill coefficient near unity (fig. 2) indicates, that the effects of these ligands are not only additive but cooperative.

The phenomena observed parallel the effects of muscarinic and histaminic receptor activators on the effect of allosteric activators on the excitable membrane of the electroplax [10] or on the binding of substrates to regulatory enzymes [13, 14]. It is therefore concluded, that recognition sites for MeCh or histamine have more than one binding area to which 'allosteric' ligands can bind.

The action of Dibenamine seems to be related to the 'desensitization phenomenon' observed with regulatory proteins. The treatment of those proteins with thiol group reagents causes the loss of sensitivity without little or no loss of activity associated with homotropic ligand binding. The loss of sensitivity is accompanied by a loss of regulatory properties, so that the Hill coefficient approaches to one. Desensitization alters also the sensitivity to heterotropic ligand. It was presumed, that the desensitizing agents interfere with a general mechanism, accounting for indirect interaction between topographically distinct ligand binding sites [10, 13, 14]. It has been shown, that the homotropic effects of MeCh or histamine alter after a treatment with Dibenamine in the way, as it was described for the 'desensitization phenomenon' (figs. 3 and 4). The alteration of heterotropic effects has been noted for the antagonistic activity of some quaternary pyridines [8] as well as for the effect of a secondary stimulating compound (fig. 5).

The stereochemical analog of a compound to the specific agonist is not required for a more or less complete protection against the receptor inactivation by Dibenamine (TMB-4 in fig. 3, Lachesine in fig. 4). These effects can be interpreted as effects of allosteric ligands, which bind to areas different from binding sites to the agonist, but interdependent on them.

The phenomena reported here are measured by means and methods, agreeing with those, on which the theory of the 'receptor reserve' is based [2–4]. A different kinetic treatment of the results yet leads to an interpretation, which is not consistent with the assumption, that Dibenamine acts exclusively on one common recognition site for the agonist and its competitive antagonist. The action of Dibenamine seems to be dependent not on the quantity of alkylated recognition sites for specific ligands, but on the conformational state of the receptor, induced spontaneously or by a protecting ligand. Dibenamine stabilizes the receptor by an indirect mechanism in a state, which can be irreversibly activated or inactivated with respect to the specific agonist. There is no evidence, that the alkylation of sites of the cell membrane, sensitive to Dibenamine, can be prevented really by the protecting agents.

This interpretation agrees with the models of cholinergic receptors with multiple binding sites [7, 9, 10, 16]. It agrees with the proposition [9], that the muscarinic receptors of muscle cells of the guinea pig ileum have regulatory properties, analogous with the acetylcholine receptors of the excitable membrane of the electroplax, membrane bound or as a purified protein [10–12), and with the structure bound acetylcholinesterase of bovine erythrocytes [17, 18]. The proposed analogy of cholinoceptive enzymes and receptors is supported by the morphological similarity of the isolated acetylcholinesterase [19] and acetylcholine receptor protein from the excitable membrane of the electroplax [20]. The interpretation of the action of Dibenamine is contradictory to the theory of a 'receptor reserve' for muscarinic and histaminic binding sites of intestinal smooth muscle cells.

Acknowledgements

The author would like to thank Professor J.-P. Changeux for helpful comments and for revising this manuscript. She thanks the Smith Kline and French Research Laboratories, Philadelphia, Pennsylvania (U.S.A.) for the kind gift of furthrethonium ($HFurMe_3$) and Dibenamine, as well as the Gerhardt–Penick Ltd, Thornton Laboratories, Croydon (U.K.) for the generous supply with Lachesine. She is gratefull to Mr. F. Brüggemann and Mr. H. Rörig for helpful technical assistance and cooperation.

References

[1] Nickerson, M. (1956) Nature 178, 697.
[2] Stephenson, R.P. (1956) Brit. J. Pharmacol. 11, 379.
[3] Ariens, E.J., Simonis, A.M. and van Rossum, J.M. (1964) in: Molecular Pharmacology (Ariens, E.J. ed.) Vol. I, pp. 408–423, Academic Press, New York and London.
[4] Furchgott, R.F. (1966) in: Advances in Drug Research (Harper, N.J. and Simmond, A.B. eds) Vol. 3, pp. 21–55, Academic Press, New York and London.
[5] Nickerson, M. (1949) Pharmacol. Rev. 1, 27.
[6] Furchgott, R.F. (1954) J. Pharmacol. 111, 265–284.
[7] Moran, J.F. and Triggle, D.J. (1970) in: Fundamental Concepts in Drug–Receptor Interactions (Danielli, J.F., Moran, J.F. and Triggle, D.J. eds) pp. 133–176, Academic Press, New York and London.
[8] Kuhnen-Clausen, D. (1970) Toxicol. Appl. Pharmacol. 23, 443–454.
[9] Kuhnen-Clausen, D. (1973) Abstract Cc 7 of the Ninth International Congress of Biochemistry, Stockholm.
[10] Podleski, T.R. and Changeux, J.P. (1970) in: Fundamental Concepts in Drug–Receptor Interactions (Danielli, J.F., Moran, J.F. and Triggle, D.J. eds) pp. 93–119.
[11] Meunier, J.C. and Changeux, J.P. (1973) FEBS Letters 32, 143–148.
[12] Changeux, J.P. (1973) Abstract Ca 5 of the Ninth International Congress of Biochemistry, Stockholm.
[13] Monod, J., Changeux, J.P. and Jacob, F. (1963) J. Mol. Biol. 6, 306–329.
[14] Monod, J., Wyman, J. and Changeux, J.P. (1965) J. Mol. Biol. 12, 88–118.
[15] Paton, W.D.M. and Rang, H.P. (1965) Proc. Roy. Soc. B., 163, 1–44.
[16] Belleau, B. (1970) in: Fundamental Concepts in Drug–Receptor Interactions (Danielli, J.F., Moran, J.F. and Triggle, D.J. eds) pp. 121–131.
[17] Kuhnen, H. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 729.
[18] Kuhnen, H. (1973) Abstract 5n 21 of the Ninth International Congress of Biochemistry, Stockholm.
[19] Rieger, F., Bon, S., Massoulié, J. and Cartaud, J. (1973) Europ. J. Biochem. 34, 539–547.
[20] Cartaud, J., Benedetti, E.L., Cohen, J.B., Meunier, J.C. and Changeux, J.P. (1973) FEBS Letters 33, 109–113.