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Regulatory Properties and Cooperativity of Membrane Bound Muscarinic Receptors of Intestinal Smooth Muscle Cells

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The regulatory properties of the membrane bound muscarinic acetylcholine receptor (mAChR) of smooth muscles from the guinea pig ileum are indicated by the S-shaped concentration-response curve to acetyl-β-methylcholine (MeCh). Cooperativity upon receptor activation is observed as well as cooperativity of heterotropic ligands: two agonists, agonist-two antagonists and agonist-antagonist-interaction. The cooperativity depends either on the integrity of the cell membrane structure or of the receptor protein. Disordering of the membrane structure is caused by a cationic detergent and results in a loss of sensitivity and cooperativity of the mAChR for the action of MeCh. Desensitization of the mAChR results from the blockade of reactive SH-groups, but not from reduced S—S-bonds. Structural differences between the mAChR and the nicotinic receptor are discussed with respect to essential disulfide bonds and reactive sulfhydryl groups.

Membrane bound acetylcholine receptors of the skeletal neuromuscular junction — the electroplax included — and of intestinal smooth muscle cells have some common features. The concentration-response curves to cholinergic agonists are sigmoidal, whether they are observed with the endplate depolarization[1], the depolarization of the excitable membrane of eel or Torpedo electric cells[2,3] or with the contractions of longitudinal smooth muscle cells of the guinea pig ileum[4,5]. The sigmoidal shape of the concentration-response curves to the agonists indicates that cooperativity is associated with receptor activation. The effects observed after the application of cholinergic ligands resemble those of regulatory enzymes upon substrate and allosteric activator or inhibitor binding.

On the other hand, the nicotinic (niAChR) and the muscarinic (mAChR) receptors differ in some functional and structural properties. They have only few potent agonists in common but no common potent antagonists. Disulfide bonds are essential for maintaining the cooperativity of the niAChR[6] but not of the mAChR.

It is the aim of this paper to present data, which may throw some light on the regulatory properties of membrane bound ileal mAChR.

RECEPTOR ACTIVATION BY POTENT AGONISTS

All experiments have been carried out with the isolated intact ileum of the guinea pig or with its longitudinal muscle strips, by methods described previously[5,7]. Acetyl-β-methylcholine (MeCh) was used as the agonist because of its very weak nicotinic activity[8]. Hexamethonium was present in the organ bath throughout in order to prevent ganglionic stimulation.

Fig. 1 presents a cumulative concentration-response curve to MeCh on the isotonic contractions of ileal longitudinal muscle strips. The curve is S-shaped and its analysis by the double reciprocal plot yields a hyperbolic line. Therefore, the Langmuir or Michaelis-Menten equations are not applicable and the curves were analysed by the Hill equation (Fig. 2, a and b). The lines were drawn to the best least square fit of the experimental values. In Fig. 2a the Hill coefficient has been computed for the curve between 5% and 95% maximal contraction and was found to be near 2.0. This indicates cooperative effects upon receptor activation.

Despite the high correlation coefficient, the experimental values from the foot of the curve deviate clearly from the computed line. Therefore, the data were reexamined. Hill coefficients were evaluated for contractions between 5% and 25% and between 30% and 90% maximal contraction (Fig. 2b). Now a better fit of the lines with the experimental values is obtained. The correlation coefficients are increased. The Hill coefficient close to the origin of the curve of Fig. 1 is significantly greater than 2.0 and often near 3.0, pointing to high cooperativity upon receptor activation. At the agonist concentration generating 25—30% maximal response, the Hill coefficient declines to a value distinctly smaller than 2.0. The transition from high to weak cooperativity is accompanied by a slight decrease in the sensitivity or affinity of the mAChR to MeCh. Whether this transition of the cooperativity is due to a change of membrane structure upon binding of the agonist[9] and/or to changes in the ion exchange through the muscle cell membrane or to the amount of calcium ions bound to the membrane[4] is not known. However, disordering of membrane structure causes a loss of sensitivity of the mAChR to MeCh and a decrease of the respective Hill coefficients. (Fig. 3). The alteration of membrane structure derives from a treatment of the muscle strips

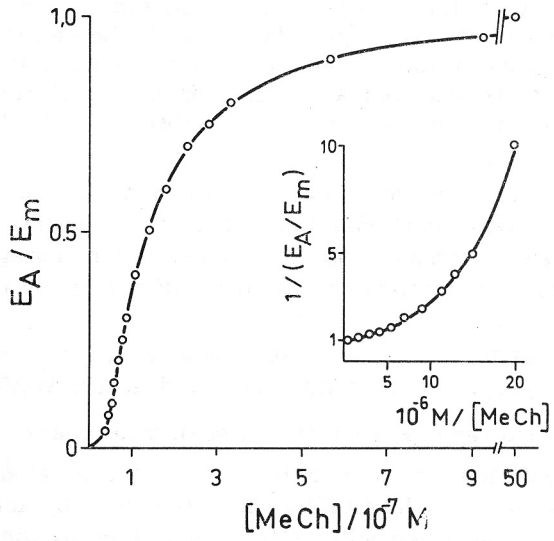

Fig. 1. Cumulative concentration-response curves to acetyl-β-methylcholine (MeCh). E_A: concentration dependent contraction of the longitudinal muscle strip of the guinea pig ileum as a fraction of the maximal effect, E_m. The inset presents the analysis of the curve by the double reciprocal plot.

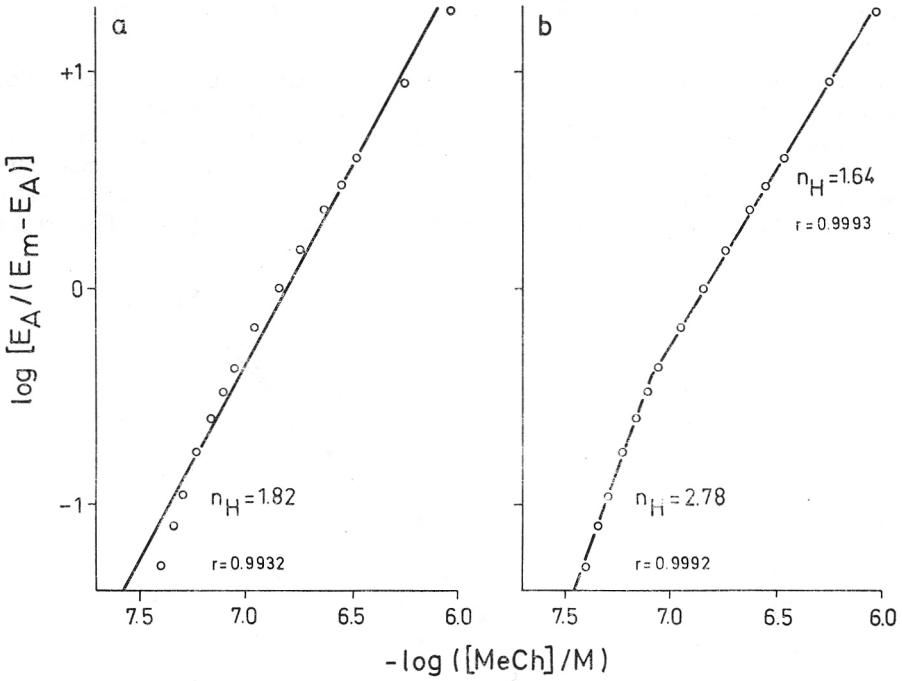

Fig. 2. Analysis of the curve of Fig. 1 by the Hill equation. Open circles: experimental values. Line: computed for the best least square fit of the experimental data. n_H: Hill coefficient. *r*: correlation coefficient. *a*: Hill coefficient evaluated for all values between 5% and 95% maximal response. *b*: Hill coefficients evaluated for values between 5% and 25% and between 30% and 90% maximal response.

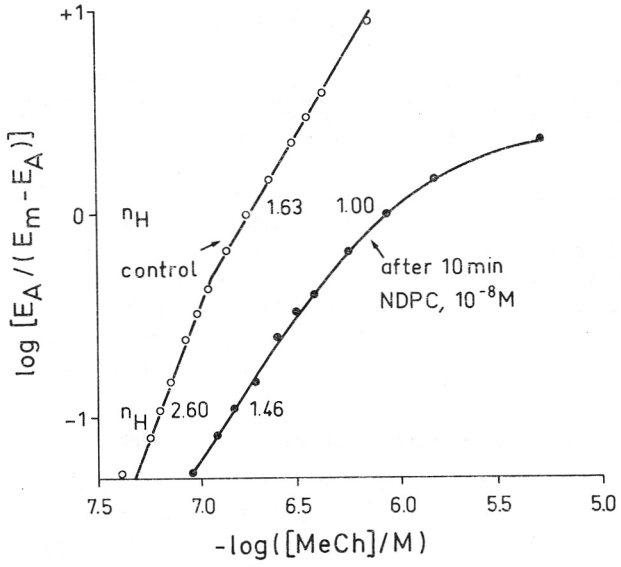

Fig. 3. Hill plot of a cumulative concentration-response curve to acetyl-β-methylcholine (MeCh) before and after the treatment of the longitudinal muscle strips with the cationic detergent N-dodecylpyridinium chloride NDPC.

with 10^{-8} M of the cationic detergent N-dodecylpyridinium chloride (NDPC) for 10 min.

Changeux and Podleski[2] found, that the cooperativity in the concentration--response curve of carbamylcholine on the electroplax disappeared when a low concentration of decamethonium (causing by itself only a slight depolarization) was present. The same effect appears when the concentration-response curve to MeCh of ileal muscles is recorded in the presence of a small concentration of 2-furfuryl-trimethylammonium chloride ($HFurMe_3$) (Fig. 4). The results

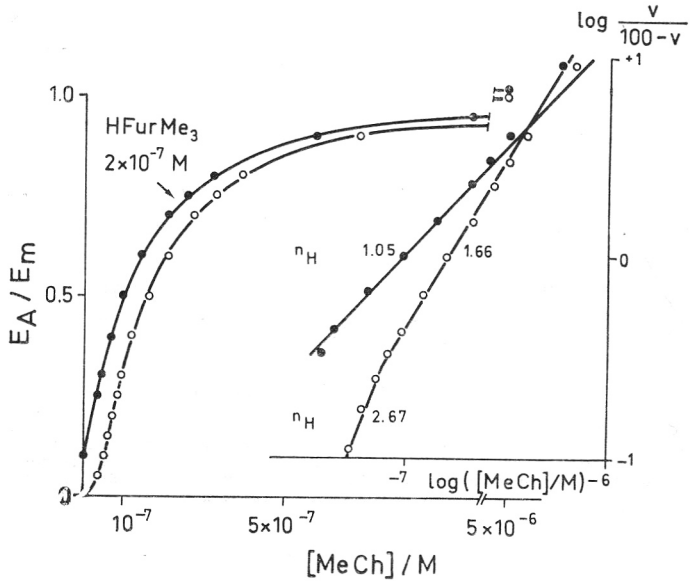

Fig. 4. The effect of the muscarinic agonist furtherthonium ($HFurMe_3$) on the shape and position of the cumulative concentration-response curve to acetyl-β-methylcholine (MeCH). The inset shows the analysis of the curves by the Hill equation. *v:* percent E_m, n_H: Hill coefficient (Redrawn from ref. 5).

resemble effects seen with regulatory enzymes and the effects of allosteric activators which convert the normally sigmoidal reaction curve into a hyperbola[10]. Such reaction requires, that both interacting ligands combine with different binding sites of the enzyme. It is therefore concluded, that recognition sites of the mAChR for agonists have more than one binding area to which receptor activators can bind[5]. The interaction of MeCh and $HFurMe_3$ would thus shift the mAChR to a conformation state of high activity and low cooperativity.

RECEPTOR INHIBITION BY REVERSIBLE ANTAGONISTS

The potent muscarinic antagonist Lachesine shifts the concentration-response curve to MeCh to the right but does not alter significantly the shape of the curve or the Hill coefficients (Fig. 5). The same holds for the action of *N*-methylatropine (not shown). Both antagonists are supposed to be specific ligands to the recognition site for MeCh on the mAChR.

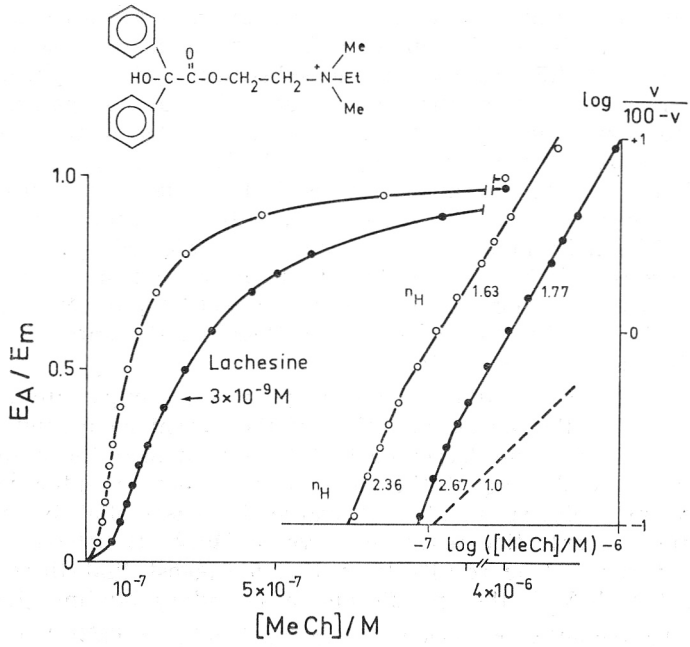

Fig. 5. The effect of the muscarinic antagonist Lachesine against acetyl-β-metylcholine (MeCh). Cumulative concentration-response curves for MeCh of longitudinal muscle strips and their analysis by the Hill equation are presented. n_H: Hill coefficient. v: percent E_m. (Redrawn from ref. 5).

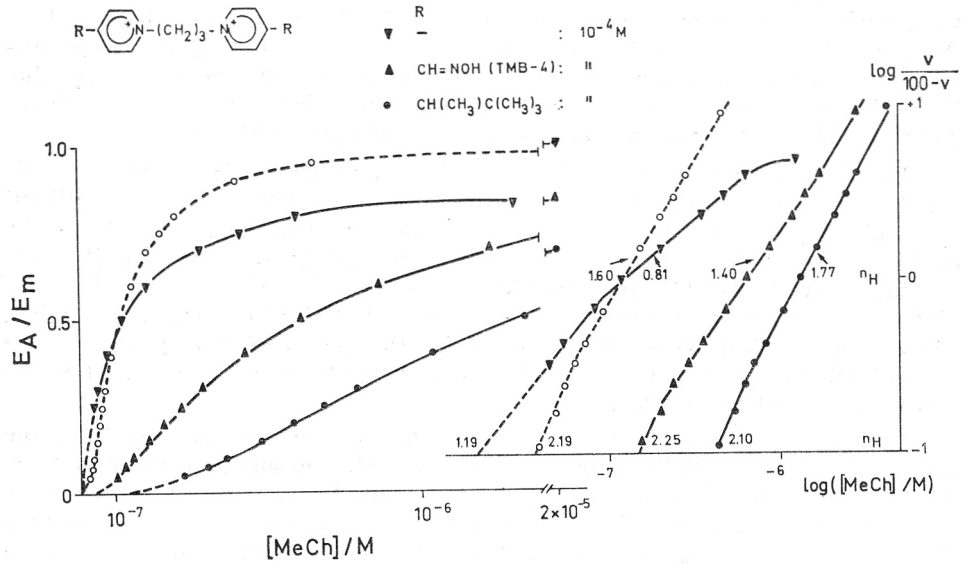

Fig. 6. Cumulative concentration-response curves to acetyl-β-methylcholine of the isolated intact guinea pig ileum. The curves were obtained in the absence (open circles, broken line) and in the presence (closed symbols, heavy lines) of bisquaternary pyridines. The inset presents the analysis of the curves by the Hill equation. n_H: Hill coefficient. v: percent E_m.

Bisquaternary pyridines are rather weak antimuscarinics when compared with Lachesine or *N*-methylatropine[7,11]. Their mechanism of action and their affinity to the mAChR depends on the substitution of the pyridinium ring. Fig. 6 presents the effects of three homologous derivatives against MeCh. The unsubstituted compound acts both as an agonist and a noncompetitive antagonist. Its stimulating effect can be supressed by Lachesine. A specific activation of the mAChR by this agent is supposed. Its antagonistic effect becomes noticeable when the agonist concentration exceeds that needed for half-maximal contraction. The noncompetitive antagonism is indicated by the decrease of maximal response of the muscle strip to high concentrations of MeCh. The shape of the concentration-response curve to MeCh approaches a hyperbola. Accordingly the Hill coefficients decrease. Possibly, the antagonistic effect is related to negative cooperativity.

The introduction of either oxime or pinacolyl groups into position 4 of the rings abolishes the activating effect of the parent compound. In the case of the dioxime TMB-4 the decreased Hill coefficient near the midpoint of the curve implies a weakening of the cooperativity upon receptor activation. In contrast, the pinacolyl substituted derivative increases slightly the Hill coefficient of the concentration-response curve to MeCh. It behaves like a weak allosteric antagonist. The results from Fig. 6 suggest that these pyridinium salts interact with both the specific and a secondary binding site for MeCh.

When the trimethylene bridge of the molecule of TMB-4 is replaced by an ether bridge, the dioxime Toxogonin results (Fig. 7). In earlier experiments[11] Toxogonin and *N*-methylatropine were combined in various serial concentrations against carbamylcholine or arecaidin-ethylester. It has been supposed that the combined antagonists should provoke effects equal to the addition of each antagonist's effect at its own concentration. However, these effects were smaller, equal or greater than expected. The differences depended on the concentration of either antagonist as well as on that of the respective agonist. Because it could not be excluded, that the results partially were due to the high nicotinic activity of both agonists, the combined activity of Toxogonin and *N*-methylatropine was reinvestigated against MeCh.

Fig. 7 demonstrates the Hill plot of concentration-effect curves of *N*-methylatropine against one single concentration of MeCh, with or without the addition of one dose of Toxogonin. The low Hill coefficient from the control curve of Fig. 7 points to a weak cooperativity upon the simultaneous binding of *N*-methylatropine and MeCh. Possibly *N*-methylatropine does not bind exclusively to the recognition site for MeCh but also to a secondary binding site on the mAChR. This was supposed for the action of Toxogonin, too. Its concentration-effect curve against the same dose of MeCh as in Fig. 7 yields a Hill coefficient[5] near 2.0.

In Fig. 7 the earlier results as mentioned above are confirmed. In the presence of 3×10^{-5} M Toxogonin (with practically no antagonistic effect) the inhibitory effects of low concentrations of N-Methylatropine are reduced, while its higher concentrations show enhanced antagonistic effects. The combined effects of these antagonists are not simply additive but cooperative in a negative or positive sense. The positive cooperative effect is independent of the dose of Toxogonin, as can be seen from the lower part of the curve, made in the presence of 10^{-3} M of this compound (Fig. 7).

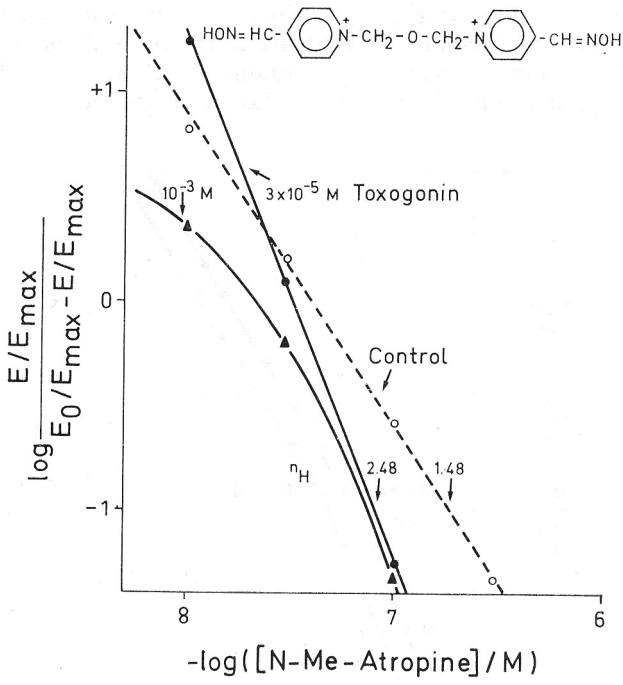

Fig. 7. Hill plot of concentration-effect curves of N-methylatropine against one single concentration (10^{-6} M) of acetyl-β-methylcholine (MeCh). Test preparation: the isolated intact guinea pig ileum. The antagonistic effects of N-methylatropine against MeCh were measured in the absence (open circles, broken line) and in the presence (closed symbols, heavy lines) of two different single doses of Toxogonin (chemical structure indicated at the top of the graph). n_H: Hill coefficient.

The phenomena described here can be interpreted as induced by allosteric ligands which bind, at least partially, to areas different from recognition sites for the agonist but interdependent from them. A mutually induced conformation change of the agonist and antagonist bindig areas is supposed. Whether the combined effects of Toxogonin and N-methylatropine against MeCh are due to changes in the sensitivity to MeCh or to one of the antagonists is not known.

DESENSITIZATION

An essential property of regulatory proteins is that when they are submitted a certain chemical treatment they loose their regulatory properties while their activity is preserved. For instance, the exposure of the isolated electroplax to the sulfhydryl reagent p-chloromercurybenzoate (PCMB) or to the disulfide reducing agent dithiothreitol (DTT) results in an increase in the half-maximal concentration of carbamylcholine and a decrease in the Hill coefficient. There is no great change in the maximal response after these treatments[3,12].

The ileal muscle strips were exposed for 10 min to 10^{-3} M of dithioerythritol (DTE), a disulfide bond reducing reagent. The treatment caused a

slight sensitization to MeCh (Fig. 8) without any loss of cooperativity. The effect is irreversible by washing. In contrast to the niAChR of the electroplax, the mAChR of the ileum apparently contains, if at all, only few disulfide groups. They are not essential for the regulatory properties of the mAChR.

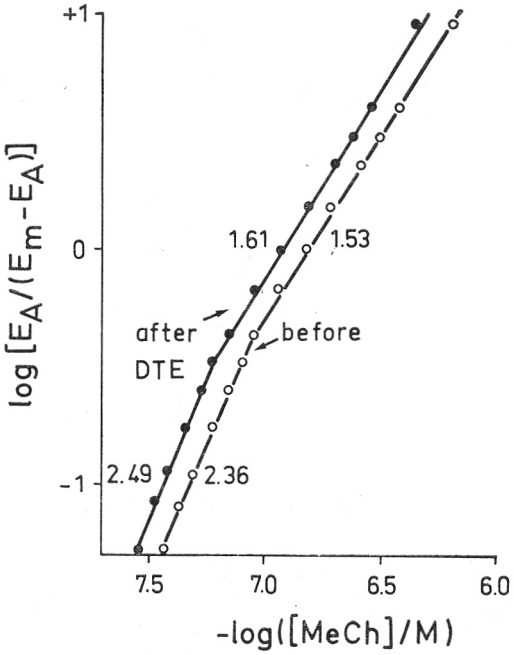

Fig. 8. Hill plot of cumulative concentration-response curves to acetyl-β-methylcholine (MeCh) of ileal longitudinal muscle strips, before and after the treatment with 10^{-3} M of the disulfide bond reducing reagent dithioerythritol (DTE). The respective Hill coefficients are indicated.

On the other hand, the muscle strips were desensitized for MeCh by a treatment with the sulfhydryl reagent PCMB (5×10^{-5} M) for 5 min (Fig. 9). The desensitization increases slowly as a function of time within 30 to 40 min. The concentration-response curve to MeCh approaches a hyperbola. The maximum response is preserved for 80%. 1.5×10^{-5} M PCMB approximately produces equal effects on the increase in the half-maximal concentration of MeCh and the decrease in the maximal response as shown in Fig. 9. However, the cooperativity is less affected. Because a 10 to 30 times smaller concentration of PCMB is needed for equieffective desensitization as for the niAChR of the electroplax[12], seemingly the mAChR contains more reactive sulfhydryl groups than the niAChR.

In some attempts made to protect the mAChR against the irreversible desensitization by PCMB, successful results were obtained with TMB-4 but not with the specific ligand Lachesine. This parallels to the observations of Podleski and Changeux[3], that d-tubocurarine, a specific ligand of the niAChR of the electroplax, did not significantly protect against the action of PCMB. In the case of the mAChR, PCMB does not seem to act directly at the level

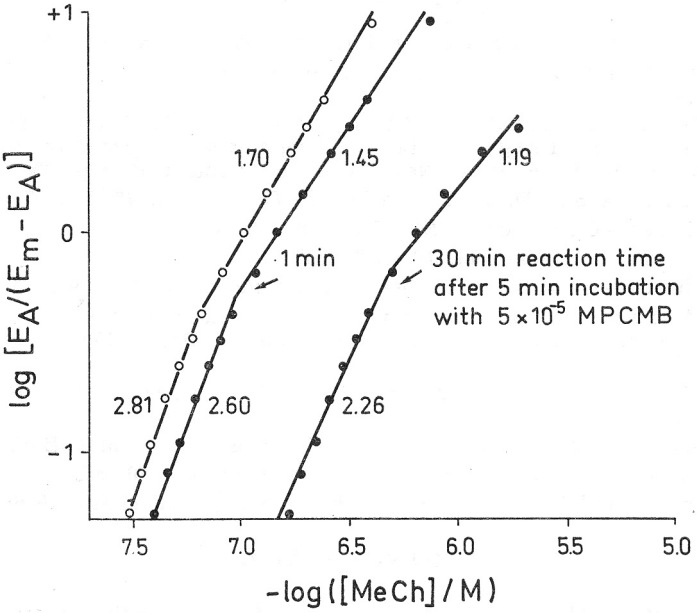

Fig. 9. Hill plot of cumulative concentration-response curves to acetyl-β-methylcholine (MeCh) of ileal longitudinal muscle strips, before (open circles) and after (closed circles) the treatment with the sulfhydryl reagent *p*-chloromercurybenzoate (PCMB). The respective Hill coefficients are indicated.

of the MeCh binding site but presumably interferes with a topographically distinct mAChR-site, sensitive for compounds like TMB-4. Both sites are supposed to be linked by indirect interactions.

CONCLUSION

The results presented here were attempted to relate drug-receptor interactions of the mAChR to comparable processes at the level of membrane bound niAChRs or to regulatory enzymes. It has been shown, that receptor activation follows sigmoidal kinetics. Chemical treatment causes desensitization for the specific agonist by an indirect mechanism. Cooperativity is associated with both receptor activation and with heterotropic ligands (agonists as well as antagonists) effects. Both cholinergic receptors have a strong relationship together. They differ, however, in some structural properties. Disulfide bonds and reactive sulfhydryl groups are essential features for the maintenance of the regulatory properties of the niAChR whereas only the reactive SH-groups determine the regulatory properties and the cooperativity of the mAChR. It is supposed, that these differences determine the specifities for the different stimulating agonists and for the antagonists.

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DISCUSSION

I. Silman:

(a) I do not think that treatment with 1 mM dithioerythritol cannot be taken as evidence that there are no disulfide bonds in the muscarinic receptor. (b) Can the effect of p-chloromercuribenzoate be reversed by thiol reagents?

D. Kuhnen-Clausen:

(a) I did the experiments in order to compare the properties of nicotinic and muscarinic receptors. All I can say is that it seems to me that the muscarinic receptor has no S—S-groups essential for the activity of the specific muscarinic recognition site, as has been demonstrated for the activity of nicotinic receptors by Karlin and Bartels. (b) I do not know yet, because the experiments are in progress.

SAŽETAK

Regulatorna svojstva i kooperativnost muskarinskih receptora glatkih mišića crijeva vezanih za membrane stanica

Dida Kuhnen-Clausen

Za regulatorna svojstva muskarinskog acetilkolinskog receptora (mAChR) vezanog za membrane glatkih mišića ileuma zamorca, karakterističan je sigmoidni oblik krivulje zavisnosti o koncentraciji acetil-β-metilkolina (MeCh). Utvrđena je kooperativnost nakon aktivacije receptora kao i kooperativnost heterotropnih liganada: interakcija dva agonista, agonista i dva antagonista te agonista s antagonistom. Kooperativnost zavisi ili o integritetu strukture stanične membrane ili o receptorskom proteinu. Promjene u strukturi membrane uzrokovane kationskim detergentom rezultiraju gubitkom osjetljivosti i kooperativnosti mAChR za MeCh. Smanjenje osjetljivosti mAChR posljedica je blokiranja SH-skupina, a ne redukcije S—S-vezova. Prodiskutirane su strukturne razlike između mAChR i nikotinskog receptora u odnosu na S—S-vezove i reaktivne sulfhidrilne skupine.

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