The Muscarinic Receptor of Chick Embryo Cells: Correlation between Ligand Binding and Calcium Mobilization

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ABSTRACT In this report we characterize muscarinic cholinergic receptor on embryonic cells. We established dose-response curves by fluorometric measurement of Ca²⁺ mobilization in cell suspensions of whole chick embryos stage 23/24. Ca²⁺ mobilization was quantitated by standardization of chlorotetracycline (CTC) fluorescence changes after stimulation with muscarinic agonists. We determined ED₅₀ values for the agonists acetylcholine and carbachol as 3.4 × 10⁻⁶ and 2.7 × 10⁻⁵ M, respectively. Pilocarpine and oxotremorine were found to act as reversible competitive antagonists with inhibition constants (Kᵢ) of 5.0 × 10⁻⁶ and 1.4 × 10⁻⁴ M, respectively. Bethanechol, which induced only 23% of the maximal effect obtained by acetylcholine, was a partial agonist with an ED₅₀ of 4.8 × 10⁻⁴ M. Its antagonistic component is expressed by an inhibition constant of 1.9 × 10⁻⁴ M. In parallel, binding studies were performed in a competition assay with [³H]-quinuclidinylbenzilate. For the agonists acetylcholine and carbachol, binding parameters were best fitted by a “two binding-sites model.” Comparison with dose-response curves indicated that Ca²⁺ mobilization was triggered via the high-affinity binding site. The inhibition constants of antagonists derived from the shift of dose-response curves corresponded to the fitted KD values of the binding studies when a “one binding-site model” was applied. Combination of dose-response and binding data showed close proportionality between receptor occupancy and calcium mobilization. No spare receptors were present.

Undifferentiated cells of the chick limb bud possess a muscarinic cholinergic receptor (1) which is assumed to be part of an embryonic cholinergic system that is expressed in undifferentiated cells during distinct phases of morphogenesis (2, 3). In a preceding publication, we described intracellular Ca²⁺ mobilization upon stimulation of the receptor (4). Ca²⁺ mobilization was measured in a spectrofluorometric assay with chlorotetracycline (CTC) (5, 6). On addition of muscarinic agonists, the cells responded with a fluorescence decrease. The reaction was blocked by muscarinic antagonists. Nicotinic drugs were ineffective.

For further characterization of the muscarinic receptor on embryonic cells, dose-response curves have to be established.

Intracellular Ca²⁺ mobilization is a biological effect that can be used for this purpose. In the present study, to quantify CTC fluorescence changes, we standardized the fluorometric measurements and improved data processing. The dose-response curves have to be related to receptor occupancy. Since ligand affinities in homogenate are different from those in cell suspensions, our binding data from homogenate (1) cannot be used for discussion of dose-response curves. In particular, binding of muscarinic agonists in homogenate is influenced by incubation conditions (7) and concentration of metabolites such as guanosine triphosphate (8). Therefore, in the present study, we determined binding of muscarinic ligands in cell suspensions in parallel to the fluorometric measurements. A binding assay with [³H]-quinuclidinylbenzilate (QNB) was established. Instead of the filter assay used for homogenate, we separated bound and free radioactivity by centrifugation. In contrast to the previous study in which cell suspensions from chick limb buds of stage 23/24 were used, we isolated...
the cells from whole embryos stage 23/24 in order to obtain the large numbers of cells necessary for serial measurements.

MATERIALS AND METHODS

Drugs and Chemicals: Enzymes and Dubecco’s modified Eagle’s medium (NaHCO₃ omitted, 20 mM HEPES supplemented) were provided by Boehringer GmbH (Mannheim, Federal Republic of Germany [FRG]). The other chemicals were purchased from the following sources: tritium-labeled (32 Ci/mmol) and unlabeled QNB (Radiochemical Centre, Amersham, U.K.), atropine sulphate (Ciba AG, Darmstadt, FRG), pirenzepine dihydrochloride (Thomae, Biberach, FRG), acetylcholine chloride, bethanechol chloride, oxotremorine, carbaryl chloride (carbamylcholine chloride), pilocarpine hydrochloride, chlorotetraclycine hydrochloride, and physostigmine sulphate (Sigma Chemie GmbH, München, FRG). Dextinamide and levetimide are gifts from Janssen GmbH (Neuss-Rosellen, FRG).

Fluorometric Measurements: Cell suspensions of whole chick embryos stage 23/24 were prepared by enzymatic digestion according to the procedure outlined in a previous publication (4). Cells were stored up to 5 h in tissue culture medium (Dubecco’s modified Eagle’s medium). Before loading the cells with CTC, we transferred them into Hanks’ solution (1.26 mM Ca²⁺) without NaHCO₃, that contained 20 mM HEPES, pH 7.3, at 37°C. For dose-response curves, up to five matched probes were prepared from a single batch of cells. The cell suspensions were prewashed with CTC (20 μM) in a roller culture apparatus (six turns per minute) at 37°C for 20 min. Fluorometric measurements were performed in a Yobin Yvon spectrofluorometer JY 3 D as described previously (4). In experiments using acetylcholine for stimulation, physostigmine (1 μM) was added before stimulation.

Binding Assay: Ligand binding in cell suspension was measured by a centrifugation assay using [³H]QNB as the specific ligand for muscarinic binding sites. Two incubations with a total volume of 1,100 μl each were prepared, the first (incubation 1) containing [³H]QNB (0.4 nM) alone, the second (incubation 2) containing [³H]QNB (0.4 nM) and radioinert atropine in excess (1 μM). Reagents and cell suspensions were prepared in Hanks’ solution without NaHCO₃ containing 20 mM HEPES, pH 7.3, at 37°C. Cell concentration varied between 5 × 10⁶ and 9 × 10⁶ cells/ml (molar concentrations of specific binding sites [B₂] between 30 and 60 pM). Incubation was performed in Eppendorf microvials in a shaking water bath (37°C, 75 cycles per minute). After an incubation period of 1.5 h, free and bound radioactivity were separated by centrifugation using a Beckman centrifuge 5414. Kinetic incubation period of 1.5 h, free and bound radioactivity were separated by centrifugation for 1 min at 7,000 g in an “Eppendorf centrifuge 5414.”

Parameter Fitting

For parameter fitting we used the nonlinear least squares regression program BMDP (BMDP statistical software, University of California, 1981) on a UNIVAC 1100/80 computer (Rechenzentrum der Universität Tübingen). Confidence limits were computed as “asymptotic standard deviations” (BMDP manual, 1981). Concentrations were internally transformed to logarithms prior to fitting. Therefore, confidence limits of parameters with the dimension “concentrations” (BMDP manual, 1981). Concentrations were internally transformed to logarithms prior to fitting. Therefore, confidence limits of parameters with the dimension “concentrations” (e.g., dissociation constants) sometimes appear unsymmetrical. The goodness of fit of different models to the same data can be compared by the mean square error (MSE) of the fit. The MSE of a fit is equal to the minimal residual sum of squares divided by (n - p), where n is the number of cases and p is the number of independent parameters. Most of the figures, including the fitted curves, were plotted with the DISSPLA software system (Integraled Software Systems Cooperation, San Diego, CA) on a Calcomp plotter connected to the UNIVAC 1100/80.

Dosage-Response Curves

Parameters were fitted by assuming one class of noninteracting receptors and proportionality between occupancy and effect (9).

$$I_{\text{cell}} = I_T - I_B$$  \hspace{1cm} (3)

$$\Delta I_{\text{stand}} = \Delta I_{\text{max}} \cdot [A]/(ED_{50} + [A])$$  \hspace{1cm} (7)

where $\Delta I_{\text{stand}}$ the standardized fluorescence decrease (Eq. 4), is the dependent variable and the agonist concentration [A] is the independent variable. The parameter $\Delta I_{\text{max}}$ signifies the maximal effect and $ED_{50}$ is the agonist concentration yielding 50% of maximal effect.
Addition of a reversible competitive antagonist (I) before stimulation resulted in a shift of the dose-response curve to the right (see Fig. 3). From EDso values of shifted (EDso') and unshifted (EDso) curves, the Ki value of the antagonistic substance can be calculated. Under the assumptions of Clark's model (9), Ki is identical to the dissociation constant of the antagonist (10):  

\[ Ki = \frac{[I] \cdot EDso}{(EDso' - EDso)}. \]  

(8)

### Saturation Studies

Specific binding of [3H]QNB was nonlinearly fitted to the saturation function (9) of a "one-site model" with the parameters Bmax (total concentration of specific binding sites) and Ko (dissociation constant):  

\[ [R - 3HQ] = \frac{B_{max} \cdot [3HQ]}{(Ko + [3HQ])}. \]  

(9)  

[3HQ], the equilibrium concentration of free [3H]QNB, is measured as F, in the supernatant of incubation 1, and [R - 3HQ], the equilibrium concentration of specifically bound [3H]QNB, is calculated as Dcorr according to Eq. 6.

### Competition Curves

The data input of one competition experiment consisted of 12 triplets of values: [A], [3HQ], and [R - 3HQ]. The concentration of competitor A was known and [3HQ] and [R - 3HQ] were measured as F, and Dcorr. We avoided systematic and random errors due to various concentrations of total and free [3H]QNB in the individual incuba-tions by measuring the concentration of free [3H]QNB individually in each incubation. Competition data were fitted to three models: The first model assumes the competition of [3H]QNB and an unlabeled ligand (A) for a single population of noninteracting binding sites (one-site model):  

\[ [R - 3HQ] = \frac{B_{max} \cdot [3HQ]}{(Ko + [3HQ]) + [A] \cdot Ko/Ka}. \]  

(10)  

Ko (the dissociation constant of [3H]QNB derived from saturation studies) has a fixed value (Ko = 10^-10 M; see Table III), [A] is the concentration of competitor and Ka is its dissociation constant. The fitting procedure creates values for the free parameters Bmax, and Ko.

The second model describes the displacement of [3H]QNB by unlabeled ligand which has different affinities for two noninteracting sites (two-sites model):  

\[ [R - 3HQ] = \frac{R\alpha \cdot [3HQ]}{Ko + [3HQ] + [A] \cdot Ko/K\alpha} + \frac{R\beta \cdot [3HQ]}{Ko + [3HQ] + [A] \cdot Ko/K\beta}. \]  

(11)  

Parameter estimates were created for the concentrations of binding sites showing high affinity (R\alpha) and low affinity (R\beta) towards the competitor and for the respective dissociation constants Ko and K\alpha.  

The third model is derived from the equation of Hill (11) and, in contrast to the models mentioned above, is not a molecular model when the Hill coefficient nH is not an integer. It gives a phenomenological description of the [3H]QNB binding if the competitor binding demonstrates cooperativity (phenomenological cooperativity model):  

\[ [R - 3HQ] = \frac{B_{max} \cdot [3HQ]}{Ko + [3HQ] + [A] \cdot Ko/K'}. \]  

(12)  

(K' is a constant without physiological meaning.)

### RESULTS

**Tracing of Ca\(^{2+}\) Mobilization by CTC Fluorescence**

Cell suspensions loaded with CTC responded to the addition of muscarinic cholinergic agonists with a fluorescence decrease. This decrease indicates receptor-mediated Ca\(^{2+}\) mobilization and was documented by continuous tracing in a spectrofluorometer (Fig. 1). The fluorescence intensity is given in arbitrary units which depend on the actual setting of the spectrofluorometer. After addition of a submaximal dose of acetylcholine, total fluorescence (IT1) decreased within 4 min and stabilized on a new plateau (IT2). IT1 and IT2 were determined graphically. The fluorescence decrease was obtained by subtraction (\(\Delta IT2 = IT1 - IT2\)). A second stimulation with a higher dose resulted in a further decline of fluorescence intensity (\(\Delta IT4\) in Fig. 1). After correction of the dilution effect, the drug-induced component of the total fluorescence changes (\(\Delta IT2\)) was standardized according to Eq. 4. The maximal drug-induced fluorescence decrease was \(\sim 15\%\) of cellular fluorescence.

**Reproducibility**

The standardized fluorescence changes \(\Delta IT2\) (Eq. 4) measured in different matched probes of one cell preparation (intra-assay variability) showed a variability of cv = 7% (n = 5; cv: coefficient of variation). The \(\Delta IT2\) values obtained in different preparations of comparable cell suspension (inter-assay variability) revealed a variability of cv = 12% (n = 49). The respective variabilities of the \(\Delta IT2\) values (Eq. 2) that were not standardized amounted to cv = 12% (intra-assay variability) and cv = 38% (inter-assay variability).

**Establishment of Dose-Response Curves**

In the experiment of Fig. 1, a first addition of a relatively low concentration of acetylcholine resulted in a submaximal...
response. A second stimulation with a higher dose of acetylcholine triggered an additional effect. After the maximal response had been reached, no further reaction was obtained. The dose-response curves described below were established by evaluation of such stepwise stimulation. Three to four additive stimulations could be measured in one probe because the plateau phase lasted only for ~20 min. If more gradations were necessary for a dose-response curve, matched cell suspensions were used.

Reversibility of Reaction

The fluorescence decrease triggered by muscarinic agonists was found to be reversed by the addition of an antagonistic drug. In the experiment shown in Fig. 2, after addition of acetylcholine, the fluorescence was reversed by pilocarpine. As described before (4), in cell suspensions from chick limb bud and from whole chick embryos, pilocarpine antagonizes the acetylcholine-induced Ca²⁺ movement. Reaction and reversal can be repeated several times with increasing concentration of agonists and antagonists. In this respect, pilocarpine and atropine show the same antagonistic behavior. After repetitive stimulation and reversal, the sum of the responses to agonists surmounted the maximal response obtainable without antagonistic reversal. The experiment demonstrated reversibility of ligand binding and repeatability of the drug effects.

In contrast to the fluorescence decrease which lasted 4 min, reversal of the fluorescence decrease by antagonistic substances took ~8 min. In the experiment shown in Fig. 2, pilocarpine was used as the antagonistic substance because its dissociation from the receptor is fast enough to allow further stimulation. For the third reversal (Fig. 2), atropine had to be used, since it is technically impossible to obtain the necessary excess of pilocarpine.

Dose-Response Curves

Fig. 3 shows dose-response curves of the agonists acetylcholine and carbachol and of the partial agonist bethanechol. The standardized effect \( \Delta I_{\text{stand}} \) is depicted against the agonist concentration. The effects were measured as indicated in Fig. 1 and standardized according to Eq. 4.

With acetylcholine and carbachol a full effect was obtained, whereas bethanechol triggered only about one fourth the maximal response obtainable with the full agonists. Table I summarizes the data of several experiments in which dose-response curves were established. The \( ED_{50} \) of carbachol was one order of magnitude higher than that of acetylcholine. The partial agonist bethanechol had the highest \( ED_{50} \). As outlined in the previous study, oxotremorine and pilocarpine showed no agonistic effect in this system (4).

Influence of Reversible Competitive Antagonists on Dose-Response Curves

Presence of the antagonistic substance pilocarpine in the assay reduced the sensitivity of acetylcholine: Higher concentrations of agonist had to be used to achieve the same effect and the dose-response curve of acetylcholine was shifted to the right (Fig. 3).

From such measurements we were able to determine the \( K_i \) values of the antagonists in question according to Eq. 8. The results for five antagonists are shown in Table II. Because of
the antagonistic component of the partial agonist bethanechol, the $K_i$ of this substance could be determined in the competitive assay for antagonists. The $ED_{50}$ value of bethanechol as agonist and its $K_i$ value as antagonist showed similar values ($4.8 \times 10^{-4}$ M and $1.9 \times 10^{-4}$ M).

According to Furchgott (10, 12), there are two groups of competitive antagonists: "reversible" and "irreversible." The results described above indicate that atropine, pirenzepine, oxotremorine, pilocarpine, levetimide, and bethanechol behave as reversible competitive antagonists in our system.

**Influence of Irreversible Competitive Antagonists**

As shown in Fig. 4, preincubation of the cell suspension with increasing concentrations of QNB led to a reduction of the maximum effect inducible by acetylcholine. $1 \times 10^{-10}$ M QNB reduced the maximal response to 50%. The $ED_{50}$ of acetylcholine within the dose-response curves did not change. Therefore, in this context, the antagonist QNB has to be classified as slowly dissociating and thus a practically irreversible competitive antagonist (10, 12). The antagonist dexetimide showed the same behavior, having no effect on the $ED_{50}$ of agonists but reducing the maximum effect (data not shown).

**Binding Studies with $[^3H]$QNB**

To correlate the effects of muscarinic drugs on $Ca^{2+}$ mobilization with occupancy of the muscarinic receptor, we performed binding studies in cell suspensions. The cell suspensions of whole chick embryos were identical to those used for CTC fluorescence measurements.

The cell suspensions were incubated with the specific muscarinic ligand $[^3H]$QNB for 90 min at 37°C. Separation of cell-bound and free radioactivity was performed by rapid centrifugation. To discriminate specific and nonspecific binding, we performed a second incubation with an excess of unlabeled atropine. Specific binding of $[^3H]$QNB to the cells was calculated according to Eq. 6. The intra-assay variability of the $D_{corr}$ values was determined as $cv = 3.4\%$ ($n = 12$).

**Saturation Studies**

Fig. 5 shows the determination of specific binding. The curve marked by solid squares gives the total bound radioactivity in incubation 1 with increasing concentrations of $[^3H]$QNB. The curve marked by solid circles gives the linear increase of nonspecific binding as determined in incubation 2. The specifically bound radioactivity (open triangles) shows saturation characteristics.

In the competition studies the radioinert ligands in question were tested. In Fig. 6, binding data of three saturation experiments are transformed and depicted as Scatchard plots (13). Intersection with the abscissa indicates specific binding capacity $B_{max}$ and the slope indicates the affinity of the specific binding sites for $[^3H]$QNB. All three plots are linear, which indicates a single class of noninteracting specific binding sites. Parameter estimates are given in Table III.

The first saturation experiment of Fig. 6 was performed with a cell suspension from limb buds that at stage 23/24 consists only of undifferentiated cells; the other two experiments used cells of whole chick embryos of the same stage. In the experiment with limb buds, a lower cell concentration was obtained leading to a lower $B_{max}$ value. In addition, the number of binding sites per cell in limb bud was lower than in cell suspensions of whole chick embryos (Table III). The essential feature of the receptor, its affinity, was identical in both preparations.

**Competition Studies**

In the competition studies the radioinert ligands in question were tested. For each of the three experiments, the competition was evaluated by calculating the $IC_{50}$ value as the concentration of the competing ligand required for 50% inhibition of the binding of $[^3H]$QNB to the cell suspension. The $IC_{50}$ values were calculated according to Eq. 8, where the $K_i$ is the value of the agonist and the $ED_{50}$ is the value of the competitive antagonist. $K_i$ values were calculated according to Table II.

**TABLE II**

| Substance          | $K_i$ Values (mol/liter) | No. of independent determinations |
|--------------------|--------------------------|-----------------------------------|
| Atropine           | $0.8 \times 10^{-9}$     | 7                                 |
| Pirenzepine        | $1.6 \times 10^{-7}$     | 2                                 |
| Oxotremorine       | $1.4 \times 10^{-6}$     | 3                                 |
| Pilocarpine        | $8.8 \times 10^{-4}$     | 3                                 |
| Levetimide         | $1.9 \times 10^{-4}$     | 4                                 |

The $K_i$ value is a measure of the antagonistic potency of the respective competitive antagonist. $K_i$ values were calculated according to Eq. 2, where the $ED_{50}$ is the value of the test agonist and the $ED_{50}$ is the value of the competitive antagonist. $K_i$ values were calculated as $mean \pm range$ (10, 50% confidence interval).

*Mean values calculated as means of log $K_i$. 

**FIGURE 5** Chick embryo cells were incubated with increasing concentrations of the ligand $[^3H]$QNB. In incubation 2 (incub. 2) specific binding was suppressed by an excess of radioinert atropine. Nonspecific (nonspec.) binding increased proportionally with $[^3H]$QNB concentration. In incubation 1 (incub. 1), in addition, specific (spec.) binding occurred. Specific binding was calculated from the difference of both curves.

**FIGURE 4** Dose-response curve of acetylcholine (A.C.H.) after equilibration with various concentrations of the antagonist QNB. Increasing QNB concentrations led to a reduction of the maximal inducible effect. STAND, standard, L, liter.
were added to incubation 1 and 2. From the paired incubations the remaining specific binding of [3H]QNB was calculated. In the resulting competition curves (Fig. 7), three groups of ligands could be distinguished. The first group comprises the antagonists QNB, dexetimide, and atropine. The IDso (concentration of competitor that reduces specific [3H]QNB binding to 50%) values were 10^{-10}, 10^{-9}, and 10^{-8} M, respectively. The second group comprises the antagonist drugs pirenzepine, oxotremorine, levetimide (not depicted in Fig. 7), and pilocarpine and the partial agonist bethanecol. The respective IDso values ranged between 10^{-6} and 2 \times 10^{-3} M. The third group comprises the fully efficacious agonists acetylcholine and carbachol. The IDso of the agonists ranged between 2 \times 10^{-4} and 2 \times 10^{-3} M. In contrast to the steep competition curves of the antagonists, those of the agonists were flat.

Tables IV + V give the parameter estimates fitted according to the one binding-site model (Eq. 10), the phenomenological cooperativity model (Eq. 12), and the two binding-sites model (Eq. 11) as described in Data Analysis.

For antagonists, the one-site model gave a good fit which was not improved by the phenomenological cooperativity model, as indicated by the MSE (Table IV). For agonists, the fit was considerably improved. Therefore, for agonists the two binding-sites model was applied (Table V).

FIGURE 6 Scatchard transformation of three saturation studies results in linear plots. Although the number of binding sites in limb bud cells was less than in cell suspensions of whole embryos, the receptor affinities were the same, as indicated by similar slopes. Spec., specifically. L, liter.

DISCUSSION

In this paper we describe a muscarinic cholinergic receptor in cell suspensions of the 3½-d chick embryo (stage 23/24). We found that stimulation of the receptor leads to intracellular Ca^{2+} mobilization which is visualized fluorometrically by changes of CTC fluorescence.

In a previous study (1) we have shown that the receptor is present in the undifferentiated limb bud of the same stage. From our histochemical studies we know that in the chick embryo stage 23/24, a large number of undifferentiated cells express embryonic cholinesterase correlated to phases of morphogenesis (2). We therefore assume that the observations in cells of whole embryos reflect reactions of the muscarinic receptor on undifferentiated cells.

Fluorometric Measurements

The decrease of CTC fluorescence intensity after stimulation reflects intracellular Ca^{2+} mobilization (4-6). It is unlikely that in our experiments extracellular Ca^{2+} is involved because over a wide range of Ca^{2+} concentrations (0-4 mM) the reaction is independent of the actual extracellular Ca^{2+} level. If the cells are stimulated in Ca^{2+}-free solution in the presence of 0.1 mM EGTA, the reaction remains unchanged, only the repetitivity of the reaction is restricted. If cells are kept in Ca^{2+}-free medium for hours they lose their ability to react presumably owing to depletion of intracellular Ca^{2+} (unpublished results).

In the present experiments the CTC assay was standardized for quantitative evaluation so that dose-response curves and EDso values could be established. The pharmacologic profile of the embryonic muscarinic receptor was found to be as follows: Acetylcholine and carbachol are full agonists whereas bethanecol is a partial agonist (23% of maximal effect). The classical agonists pilocarpine and oxotremorine do not trigger Ca^{2+} mobilization in embryonic cells.

FIGURE 7 In the competition studies with [3H]QNB, the respective muscarinic ligand was added to the incubations in increasing concentrations. Maximal binding was determined without competitor and nonspecific binding in presence of 1 \mu M atropine. Only the values in the respective saturation range are shown. Curves are fitted as outlined in Data Analysis. Parameters are given in Tables IV and V. L, liter.

TABLE III

| Material          | Cell concentration | No. of data | Bmax binding sites per cell |
|-------------------|--------------------|-------------|-----------------------------|
| Chick limb bud    | 6.4 \times 10^6    | 6           | 24.2 \pm 0.5^*              |
| Whole embryo      | 9.5 \times 10^6    | 13          | 59.2 \pm 1.4^*              |
| Whole embryo      | 9.0 \times 10^6    | 19          | 52.8 \pm 1.0^*              |

Parameters were fitted according to Eq. 9 (see Data Analysis) with the BMDP software (BMDP Statistical Software, University of California, 1981) on the UNIVAC 1100/80 (Rechenzentrum der Universität Tübingen).

* Asymptotic standard deviation (compare BMDP manual, 1981).
When antagonists were added before stimulation by an agonist, two distinct patterns were observed. The first class of antagonists shifted the dose-response curve into higher concentration ranges without lowering the maximal effect and without affecting the shape of the curve (Fig. 3). According to Furchgott (10, 12), these compounds are classified as reversible competitive antagonists. The classical muscarinic antagonists atropine, pirenzepine, and levetimide belong to this group as well as the classical muscarinic agonists oxotremorine and pilocarpine. The partial agonist bethanechol also shifts the dose-response curve of a full agonist to the right. From the shift of dose-response curves, we determined the inhibitory constants ($K_i$ values; Table II).

The second class of antagonists, comprising QNB and dexetimide, led to dose-dependent diminution of maximal effect, the $ED_{50}$ remaining nearly unchanged (Fig. 4). According to Furchgott (10, 12) these compounds are classified as irreversible competitive antagonists. The term "irreversible" in this context states that the compounds of this group dissociate so slowly from the receptor sites that during the time course in which the biological effect is observed (20 min), the blockade seems to be irreversible. In our system, the half-life

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**Table IV**

Parameters According to the One-site Model and the Phenomenological Cooperativity Model

| Substance          | $K_A$ (Confidence limits) | MSE | $n_H \pm s^*$ | MSE |
|--------------------|---------------------------|-----|----------------|-----|
| QNB                | $2.9 \times 10^{-11}$     | 0.89| $0.98 \pm 0.15$| 1.0 |
| Dexetimide         | $3.6 \times 10^{-10}$     | 1.3 | $0.63 \pm 0.08$| 0.5 |
| Atropine           | $1.7 \times 10^{-8}$      | 1.4 | $0.79 \pm 0.12$| 1.2 |
| Pirenzepine        | $1.1 \times 10^{-7}$      | 3.9 | $1.31 \pm 0.39$| 4.0 |
| Ooxotremorine      | $1.8 \times 10^{-6}$      | 0.4 | $0.95 \pm 0.08$| 0.4 |
| Levetimide         | $5.4 \times 10^{-6}$      | 1.6 | $0.76 \pm 0.16$| 1.5 |
| Pilocarpine        | $8.8 \times 10^{-9}$      | 2.8 | $0.68 \pm 0.07$| 1.3 |
| Acetylcholine 1    | $3.5 \times 10^{-15}$     | 5.1 | $0.41 \pm 0.05$| 0.9 |
| Acetylcholine 2    | $2.7 \times 10^{-15}$     | 8.3 | $0.39 \pm 0.06$| 1.7 |
| Carbachol          | $2.7 \times 10^{-14}$     | 8.1 | $0.41 \pm 0.07$| 1.6 |
| Bethanechol        | $4.6 \times 10^{-14}$     | 2.0 | $0.65 \pm 0.09$| 1.1 |

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**Table V**

Parameters According to the Two-sites Model

| Substance          | $K_{RH}$ (Confidence limits) | $K_{KL}$ (Confidence limits) | MSE |
|--------------------|-----------------------------|-------------------------------|-----|
| Acetylcholine 1    | $15.3 \pm 1.6$ (53)         | $3.9 \times 10^{-6}$ (2.6-5.9 $\times 10^{-6}$) | 1.1 |
| Acetylcholine 2    | $20.9 \pm 1.9$ (52)         | $4.4 \times 10^{-5}$ (3.1-6.3 $\times 10^{-5}$) | 1.6 |
| Carbachol          | $14.6 \pm 2.0$ (44)         | $1.7 \times 10^{-5}$ (1.1-2.6 $\times 10^{-5}$) | 1.1 |
| Bethanechol        | $9.1 \pm 3.2$ (31)          | $2.6 \times 10^{-5}$ (1.0-6.9 $\times 10^{-5}$) | 0.9 |
of the QNB-receptor complex (at 37°C) is 4–5 h (unpublished data).

When added after stimulation, all antagonistic compounds including oxotremorine and pilocarpine were capable of reversing the agonist-induced Ca²⁺ mobilization. Fig. 2 illustrates that stimulation by agonist and reversal by a reversible competitive antagonist could be repeated several times in the same cell suspension. The reversibility and repeatability of the effects are strong arguments for the biological character of the reaction.

**Binding Studies**

In parallel to the fluorometric measurements, we determined the muscarinic binding sites using [³H]QNB as the ligand. Separation of bound and unbound radioactivity by centrifugation maintained the equilibrium and thus yielded unbiased values as compared with a filter assay. By measuring unbound [³H]QNB in every incubation, we were able to correct for errors due to differing amounts of unbound [³H]QNB in the individual incubations. This avoids systematic errors in the calculation of specific binding (Eq. 6) and in the parameter fits.

The Scatchard plots indicate the existence of a single population of noninteracting specific binding sites for [³H]QNB (Fig. 6 and Table III). In the competition studies the antagonists showed steep sigmoid curves whereas for the agonists acetylcholine and carbachol flat competition curves were obtained (Fig. 7). The binding data of antagonistic compounds are in agreement with the assumption of a single class of binding sites (one-site model, Table IV). The observation of a single class of noncooperating binding sites for muscarinic agonists is in accordance with results of Birdsell and Hulme (14, 15). For the agonists acetylcholine and carbachol, the phenomenological cooperativity model resulted in a distinctly better fit than the one-site model (Table IV). The Hill coefficients between 0.38 and 0.41 indicate negative cooperativity of agonist binding. This is a general finding in agonist binding of muscarinic receptors which is usually explained by assuming at least two distinct classes of noninteracting binding sites with different affinities for agonists (16, 17).

**Correlation of Ca²⁺ Mobilization and Receptor Occupancy**

The Kᵢ values of antagonists determined from the shift of dose-response curves (Table II) are in good agreement with the Kₒ values calculated according to the one-site model (Kᵢ values Table IV). In the fluorometric measurement, oxotremorine and pilocarpine were observed to behave as antagonists. In the binding studies they showed competition curves with one Kₒ and no signs of cooperativity. This binding behavior is characteristic of antagonists.

From the dose-response curves the ED₉₀ of acetylcholine were quantitated as 3.4 × ¹⁰⁻⁶ M (Table I). This value is nearly equal to the Kₒ of the high-affinity site (Kₒ in Table V) of the two-sites model. The same holds true for the ED₉₀ and the Kᵢ value of the high-affinity binding site of carbachol. We conclude that in chick embryo cells, the biological effect of Ca²⁺ mobilization is triggered via the high-affinity binding site. Accordingly, the agonistic component of the partial agonist bethanechol was expressed in the high-affinity binding site when competition data were fitted for the two-sites model (Table V). Declining pharmacological activities (Table I) correlated with declining affinities of high-affinity binding sites (Kᵢ in Table V) and with declining ratios of high- to low-affinity binding sites (Rₚ/Rᵢ in Table V). The dissociation constant of the low-affinity binding sites (Kᵢ in Table V) remained nearly constant. Thus, biological activity seems to be independent of low-affinity binding sites. Correlation between high-affinity muscarinic binding sites and Ca²⁺ influx into smooth muscle cells was described by Triggle (19). Other authors, however, relate biological responses to low-affinity muscarinic binding sites (17, 20–23).

Fig. 8 correlates the maximal Ca²⁺ mobilization that can be induced in embryonic cells by muscarinic agonists with the fraction of muscarinic receptor sites occupied by an agonist. The number of available receptor sites is varied by preincubation with the irreversible competitive antagonist QNB as shown in Fig. 4. 100% of the relative fluorescence change was obtained without QNB. Gradual blocking of binding sites by QNB led to a proportional diminution of the biological effect that can be triggered by saturating concentrations of agonists. The inducible Ca²⁺ mobilization was proportional to the number of available binding sites. No spare receptors were present.

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**Figure 8** The fraction of unblocked binding sites at a given QNB concentration (abscissa) is correlated with the maximal effect obtained by saturating agonist concentrations at the same QNB concentration (ordinate). The fluorescence changes were obtained from the dose-response curves after preincubation with various concentrations of QNB (Fig. 4). The fraction of receptor sites available at the respective QNB concentration was calculated from the dissociation constant derived from the saturation studies (Fig. 6 and Table III).
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