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A Model for the Interaction of Muscarinic Receptors, Agonists, and Two Distinct Effector Substances

Allen P. Minton[‡] and Mordechai Sokolovsky*[,§]

Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Laboratory of Neurobiochemistry, Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel Aviv 69978, Israel

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ABSTRACT: The binding of the agonist carbamylcholine to muscarinic receptors in rat heart myocytes from young and aged cultures and in rat atrial membranes has been measured in the absence and presence of GppNHp, pertussis toxin, and/or batrachotoxin. The effect of each of the added substances upon agonist binding was accounted for by a model according to which the receptor may form an equilibrium complex with agonist and either of two distinct effector substances, one of which is postulated to increase the affinity of receptor for agonist and the other of which is potulated to decrease the affinity of receptor for agonist.

While it is known that the binding of muscarinic agonists to their receptors inhibits the production of cyclic AMP, presumably through interaction with the adenylate cyclase system (Watanabe et al., 1978; Brown, 1979; Van Sande et al., 1977; Olianas et al., 1983), the molecular mechanism of interaction between muscarinic receptors and other membrane and intracellular components such as ion channels is as yet unclear [for reviews, see McKinney and Richelson (1984), Nathanson (1987), Sokolovsky et al. (1986), and Sokolovsky (1989)]. Because the addition of GTP and GTP analogues to membrane preparations has a significant effect on the binding of muscarinic agonists to receptors, the existence of agonist-mediated interactions between muscarinic receptors and one or more forms of G-protein is inferred (McKinney & Richelson, 1984; Nathanson, 1987; Sokolovsky et al., 1986; Sokolovsky, 1989). While classical antagonist binding isotherms are generally explicable in terms of a model for the binding of ligand to a single class of homogeneous sites (Nathanson, 1987; Sokolovsky, 1989), data characterizing the displacement of antagonist by agonist have usually required that two or three affinity classes of binding sites be postulated (Birdsall et al., 1980). Common to both the two- and three-affinity-class models has been the assumption that the fractional abundance of each class of site is independent of the concentration of agonist. The binding isotherm of agonist to n affinity classes of sites is then specified by n equilibrium association or dissociation constants and $n - 1$ fractional site abundances.

In previous studies of the effect of exogenously added substances (such as GppNHp)[1] on the affinity of receptor for agonist, the results have typically been analyzed by fitting a phenomenological two- or three-site-class model to the data (Berrie et al., 1979; Sokolovsky et al., 1980; Wei & Sulakhe, 1980; Burgisser et al., 1982; Ehlert et al., 1981; Harden et al., 1982; Walbroeck et al., 1982). If affinity for agonist is increased in the presence of the additive, the effect is "explained" as an additive-induced conversion of lower affinity sites to higher affinity sites; conversely, if affinity for agonist is decreased in the presence of the additive, the effect is "explained" as an additive-induced conversion of higher affinity sites to lower affinity sites (Birdsall et al., 1980; Sokolovsky et al., 1986). We feel that such "explanations" are merely parameterized descriptions of the data and provide little or no insight into the molecular mechanism(s) underlying the effect of exogenous additives upon agonist binding. The purpose of the present work is to present a mechanistic model that attempts to describe agonist binding and the effects of exogenous additives on agonist binding *in the context of receptor function*. Such a model must differ from a phenomenological description of agonist binding in at least two important ways:

(1) When phenomenological two- or three-site-class models are fit to a series of isotherms characterizing agonist binding to receptor in the presence of varying amounts of exogenous additive A, it is commonly observed that the affinities for agonist, as well as the abundances of the apparent site classes, vary substantially with the concentration of A (Sokolovsky et al., 1986; Sokolovsky, 1989). In contrast to a phenomenological approach to the definition of site classes, we adopt a mechanistic approach wherein total receptor is considered to exist as a population of substates. An individual substate of receptor may differ from other substates by virtue of intrinsic differences in polypeptide structure or by virtue of covalent or noncovalent association with other naturally occurring or

* Author to whom correspondences and reprint requests should be addressed.
‡ National Institutes of Health.
§ Tel-Aviv University.

[1] Abbreviations: BTX, batrachotoxin; 4NMPB, *N*-methyl-4-piperidyl benzilate; GppNHp, guanyl-5′-yl imidodiphosphate; PT, pertussis toxin.

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exogenously added components of the system.[2] At constant temperature, pH, and buffer composition, each substate is characterized by a number of fixed properties, among which is the affinity of that substate for various agonists and antagonists. The addition of exogenous substance A to a receptor preparation containing a mixture of substates may result in the binding of A to one or more substates of receptor. Alternatively, addition of A may modify the interaction between one or more receptor substates and one or more other components of the preparation, causing net formation or dissociation of complexes between substates of receptor and other components. The net result *in either case* is the conversion of one or more substates of receptor to other substates, which may or may not have different affinities for agonist. But since the affinity for agonist is defined to be an invariant property of a given substate under a given set of environmental conditions, the individual site binding constants in a mechanistic model are constrained to be independent of the concentration of exogenous additive.

(2) A receptor is not simply a binding site; it is also a transducing device by means of which the presence of bound agonist is communicated to other components of the cellular machinery, an initial step in the elicitation of cellular response to the presence of agonist. In our view, any mechanisitc (as opposed to phenomenological) model for agonist binding must posit an interaction between receptor and at least one other cellular constituent that is modified upon binding of agonist to receptor.

The simplest model that includes a mechanism by means of which agonist binding can modulate the interaction between receptor and another cellular component is the *floating receptor* or *simple ternary complex* model (Jacobs & Cuatrecasas, 1976), which formulates equilibrium relations in a system containing ligand, receptor, and a second membrane component termed effector that can interact preferentially with either the unliganded or liganded form of the receptor. More recently, Ehlert (1985) proposed a similar model for ligand-linked interaction between muscarinic receptors and a single G-protein, according to which the complex of receptor and G-protein (RG) has a higher affinity for agonist than receptor alone (R). Guanine nucleotides are postulated to lower the overall affinity of receptor for agonist by destabilizing the interaction between receptor and G-protein, thus converting the higher affinity RG into the lower affinity R. This model has been subsequently explored and rejected by Wong et al. (1986) and Burgen (1987) as being inadequate to accommodate muscarinic receptor binding data from their respective laboratories. In preliminary calculations, we found that the ternary complex model may be fit to the data analyzed here but only if agonist binding constants for each of the two receptor substates in this model (R and RG) are allowed to vary substantially. Since this finding is inconsistent with the first requirement of a mechanistic model (invariance of individual substate affinities), we also consider the simple ternary complex model inadequate as a mechanistic basis for interpretation of agonist binding isotherms.

The simple ternary complex model may be readily extended to the case of a receptor having ligand-modulated interactions with two distinct and independent effector substances (Minton, 1982). This *extended ternary complex* (ETC) model thus posits three rather than two receptor substates, and it will be shown that three substates with invariant affinity constants for agonist suffice to accommodate all of the data that are analyzed in the present work.

We introduce here a new interpretation of msucarinic agonist binding data based upon the ETC model. According to this model, receptor interacts with at *least* two other protein species in the membrane (two distinct membrane proteins or two distinct forms of a single membrane protein), and the interaction between receptor and *at least* one of the two other species is modulated by agonist (Sokolovsky et al., 1986; Sokolovsky, 1989). The quantitative model deriving from this postulate is fit to agonist binding data obtained from two types of membrane preparations and is found to describe a wide variety of experimental results as well as the phenomenological two- or three-site-class models with approximately the same number of freely adjustable parameters.

Experimental Procedures

Materials

All reagents employed in this study are those described earlier (Moscona-Amir et al., 1986). Batrachotoxin (BTX) was a gift of Dr. John W. Daly (Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). BTX was dissolved in ethanol to a concentration of 1 mM and added to the assay mixture immediately before use. Pertussis toxin (PT) was obtained from List Biochemicals (Campbell, CA).

Methods

Preparation of Myocyte Homogenates. Myocyte-enriched cultures (>95% myocytes) were prepared from the hearts of 1–3-day-old rats (CD strain) as described earlier (Moscona-Amir et al., 1986). Cell homogenates prepared from young cultures (5 days after plating) and aged cultures (14 days after plating) were washed three times with a modified Krebs–Henseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11 mM glucose, pH 7.4) and scraped off each dish in 1 mL of the above solution. After a 3-fold dilution in the same buffer, they were homogenized at setting 5 on an Ultra Torrax homogenizer (Ika-Werk Instruments, Dottingen, FDR) with three 15-s bursts separated by 30-s pauses.

Preparation of Atria. Homogenates were prepared as described earlier (Cohen-Armon & Sokolovsky, 1986). Adult male rats of the CD strain were maintained in an air-conditioned room at 24 ± 2 °C for 14 h under fluorescent illumination and for 10 h in darkness. Food from Assia Maabarot Ltd. (Tel Aviv, Israel) and water were supplied ad libitum. Rats aged 3–4 months and weighing 190–250 g were decapitated and their hearts rapidly removed. Atria were immersed in cold 50 mM Tris-HCl buffer, pH 7.8, containing the following mixture of protease inhibitors: 5 units/mL aprotinine, 5 μg/mL pepstatin A, and 0.1 mM phenylmethanesulfonyl fluoride. For each experiment 6–10 atria were used. Atrial tissue was washed of blood in the same buffer, minced, and homogenized at setting 7 on the Ultra Torrax homogenizer with the protocol described above. The homogenates were then centrifuged twice at 30000*g* for 20 min. The final pellet was resuspended in modified Krebs buffer (pH 7.8 at 36 °C) containing the above mixture of protease inhibitors. In experiments where BTX was used, the tissue preparation was preincubated with BTX at 36 °C for 30 min. A fresh solution of diisopropyl fluorophosphate in water was added to the homogenates to a final concentration of 150 μM. The homogenate was incubated for a further 15 min at 25 °C prior to

[2] Note that this definition of substrates is more general than the conventional definition of receptor subtypes (for example, muscarinic receptor subtypes M_1, M_2, ...), which would comprise a subset of all possible substrates.

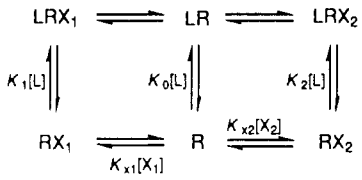
the binding assay. Protein concentration was determined according to Lowry et al. (1951), with bovine serum albumin as a standard.

Binding Assay. The binding of carbamylcholine was measured by competition against 2 (±0.5) nM [^{3}H]4NMPB (Moscona-Amir et al., 1986; Cohen-Armon & Sokolovsky, 1986). The reaction was terminated by rapid filtration through Whatman GF/C filters, followed by washing three times with the cold-modified Krebs buffer and counting radiolabel by liquid scintillation spectrometry (Packard Tri-Carb 300). Nonspecific binding was determined in the presence of 5 μM unlabeled atropine and subtracted from the total binding to obtain specific binding.

Pertussis Toxin Treatment. Pertussis toxin (final concentration 25 ng/mL) was added directly to a dish of intact cultured myocytes and the dish incubated for 12 h at 37 °C, with 5% CO_2 in the growth medium (Moscona-Amir et al., 1989).

Description of Model

We postulate that receptor R may interact with (at least) two different components called X_1 and X_2. At equilibrium, as many as three different affinity classes of receptor may coexist, corresponding to bare (uncomplexed) receptor R and the complexes RX_1 and RX_2. The affinity of bare R for agonist L is characterized by the association constant K_0, and the association constants of RX_1 and RX_2 for L are denoted by K_1 and K_2, respectively. The equilibrium constant for association of X_1 to R is denoted by K_{x1} and that for association of X_2 to R by K_{x2}. The species denoted by R may include all subtypes of muscarinic receptor that are experimentally indistiguishable on the basis of their respective affinities for a given agonist. The model is summarized in the following reaction scheme:[3]



The equilibrium concentrations of all species may be expressed as functions of the five equilibrium constants and the concentrations of free L, R, X_1, and X_2:

$$[RX_1] = K_{x1}[R][X_1]$$

$$[RX_2] = K_{x2}[R][X_2]$$

$$[LR] = K_0[L][R]$$

$$[LRX_1] = K_1[L][RX_1] = K_{x1}K_1[L][R][X_1]$$

$$[LRX_2] = K_2[L][RX_2] = K_{x2}K_2[L][R][X_2]$$

The total amount of R is given by

$$R_t = [R] + [LR] + [RX_1] + [LRX_1] + [RX_2] + [LRX_2]$$
$$= [R](1 + K_0[L]) + K_{x1}[X_1](1 + K_1[L]) + K_{x2}[X_2](1 + K_2[L]) \quad (2)$$

The total amounts of X_1 and X_2 are given by

$$X_{1t} = [X_1] + [RX_1] + [LRX_1]$$
$$= [X_1](1 + K_{x1}[R])(1 + K_1[L]) \quad (3)$$

$$X_{2t} = [X_2] + [RX_2] + [LRX_2]$$
$$= [X_2](1 + K_{x2}[R])(1 + K_2[L]) \quad (4)$$

If the constants of the model (K_0, K_1, K_2, K_{x1}, K_{x2}, R_t, X_{1t}, X_{2t}) and [L] are specified, then eqs 1–4 constitute a set of eight equations in eight unknowns that may be solved for the value of each of the species appearing in the above reaction scheme. Then the fractional saturation of receptor with agonist L is calculated from

$$y([L]) = (\text{bound L})/R_t = ([LR] + [LRX_1] + [LRX_2])/R_t \quad (5)$$

In order to simplify calculations, we introduce an alternate set of scaled constants of the model:

$$\beta_1 = K_1/K_0 \quad \beta_2 = K_2/K_0 \quad Q_1 = K_{x1}R_t$$
$$Q_2 = K_{x2}R_t \quad \alpha_1 = X_{1t}/R_t \quad \alpha_2 = X_{2t}/R_t \quad (6)$$

If the values of K_0 and the above six constants are specified, the appropriate transformations of eq 1–5 may be used to calculate y as a function of [L] (see Appendix). K_0, the β's, the Q's, and the α's thus comprise seven adjustable parameters of the model to be optimized when the model is being fit to the data as described below.

Fitting Procedures

It is assumed throughout the present work that the fractional inhibition of antagonist binding for a given agonist concentration is equal to the fractional saturation of receptor by agonist. This assumption requires that essentially all of the labeled ligand present be unbound and that the labeled ligand binds univalently to all sites in accordance with an independent single-site-class model (Chatelier, 1987). These conditions are met in all of the experimental data to which we shall refer below (Moscona-Amir et al., 1986; Cohen-Armon & Sokolovsky, 1986).

Model equations were fit to data by the criterion of least squares with a modified simplex algorithm (Press et al., 1987).[4] In order to fit the combined data with the fewest number of independently adjustable parameters, the parameters K_0, Q_1, Q_2, β_1, and β_2 were constrained to be identical for all binding isotherms for a given cell membrane (i.e., atrial or myocyte membranes). The constraints on K_0 and β_i are in accordance with our mechanstic requirement that the affinity of each receptor substate for agonist be an invariant property of the substate, and the constraints on Q_i will be justified under Discussion. *The only parameters allowed to vary between individual isotherms obtained from a given membrane type were α_1 and/or α_2.*

Results

Two distinctly different sets of experimental data were analyzed. The first set of data describes the binding of carbamylcholine to muscarinic receptors in rat myocyte membrane homogenates obtained from young (5 day) and aged (14 day) cultures in the presence and absence of the guanine nucleotide GppNHp and pertussis toxin. These data are of

[3] Equilibrium constants for the two reactions indicated on the top row of the scheme may be calculated from the five constants shown explicitly.

[4] Fitting a model to data is a procedure whereby a systematic search is made for a set of parameters, or constants of the model, such that the dependence of the dependent variable (y in this instance) upon the independent variable ([L] in this instance) calculated by the model with that set of parameter values agrees with the experimental data as closely as possible according to the criterion of least squares. If the best-fit calculation of y as a function of [L] agrees with the data to within the uncertainty of the measurement, the model is said to accommodate or be compatible with the data; if not, the model and data are adjudged incompatible. Fitting a model to data is often used as a means for determining the value of parameters that cannot be measured directly. However, we shall subsequently show that the data considered here are compatible with several qualitatively different sets of parameter values and hence do not permit us to evaluate the parameter values unequivocally.

special interest because it has been reported previously (Moscona-Amir et al., 1986, 1989) and confirmed here that in young myocytes the addition of guanine nucleotide decreases the affinity of receptor for agonist (as is usually observed in various muscarinic receptor preparations), while in aged myocytes the addition of guanine nucleotide increases the affinity of receptor for agonist.

The second set of data describes the binding of carbamylcholine to rat atrial membranes in the absence and presence of various concentrations of batrachotoxin, a persistent activator of voltage-gated sodium channels [for a review, see Catterall (1980)]. These data are of particular interest because it has been reported previously (Cohen-Armon & Sokolovsky, 1986) and confirmed here that the affinity of receptor for muscarinic agonist is increased in the presence of low concentrations of BTX and subsequently decreased in the presence of higher BTX concentrations. It has been suggested (Cohen-Armon & Sokolovsky, 1986) that the opening of sodium channels and the binding of agonists to muscarinic receptors in rat atrial membranes are coupled events that are mediated by functionally linked binding of G-proteins.

Effect of GppNHp and Pertussis Toxin on Binding of Carbamylcholine to Young and Old Cultured Rat Heart Myocytes. All of the carbamylcholine binding data, obtained from both young and old cultures, in the presence and absence of pertussis toxin and in the presence and absence of GppNHp, were fitted, to within experimental precision, by a model in which the binding of effector X_1 to muscarinic receptor is associated with an increase in affinity of receptor for agonist and in which the binding of effector X_2 to receptor is associated with a decrease in affnity of receptor for agonist. For simplicity and to reduce the number of independently variable parameters in the model, it was assumed that the parameters K_0, Q_1, Q_2, β_1, and β_2 were globally invariant and that differences in binding under different experimental conditions (i.e., young versus old myocytes, absence or presence of PT and/or GppNHp) could be accounted for by changes in α_1 and α_2. It is acknowledged that these assumptions are to some extent arbitrary; their significance will be addressed under Discussion.

We found that the model could fit the data satisfactorily with (at least) four sets of parameters reflecting different physical situations. These parameter sets are given in Table I, and one representative set of calculated isotherms is plotted together with the data in Figure 1. Isotherms calculated with other combinations of parameter values given in Table I are not plotted as they are almost indistinguishable from those shown. The possibilities reflected by these parameter sets are as follows:

Parameter Set 1: *Q_1 and Q_2 Large* ($<10^5$). In this case, both X_1 and X_2 bind stoichiometrically to R at all agonist concentrations. The receptor is functionally partitioned into three affinity classes, the abundance of which is independent of agonist binding. This model is mathematically homologous to the three-state binding models of Wong et al. (1986) and Burgen (1987). Even though this parameter set satisfactorily accommodates the carbamylcholine binding data presented here, it does not accommodate data indicating that the binding of BTX to sodium channels is modulated by the presence of muscarinic agonists (Cohen-Armon & Sokolovsky, 1986; Cohen-Armon et al., 1988). These latter data provide evidence that agonist binding to muscarinic receptors is accompanied by the release and/or uptake of at least one additional cellular component that also interacts with sodium channel in a ligand-linked fashion. Hence, we believe that this parameter set

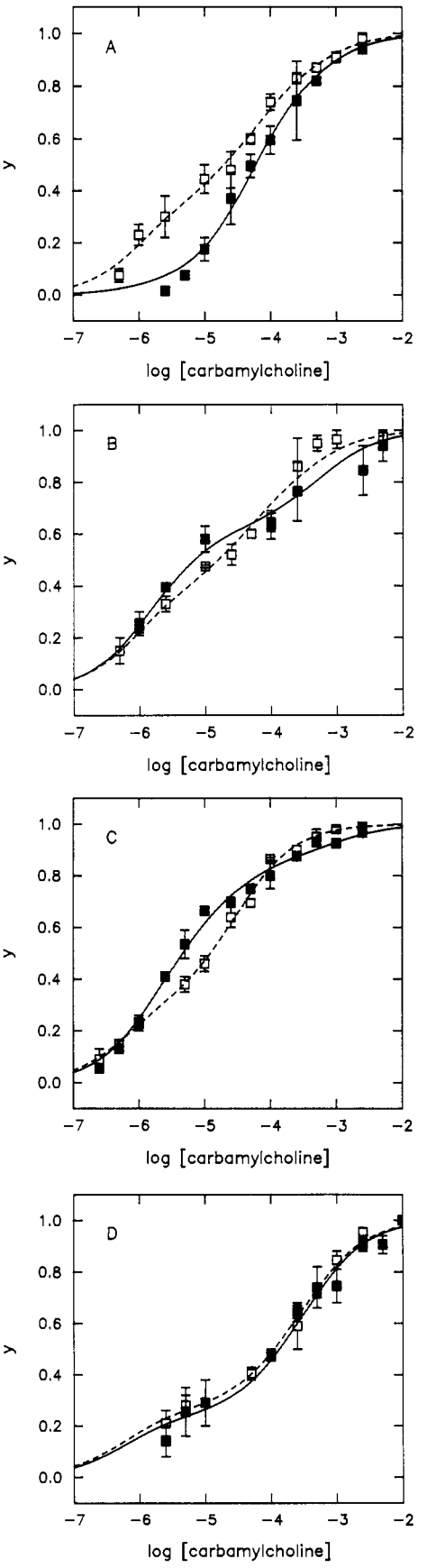


FIGURE 1: Fractional saturation (y) of rat myocyte membrane receptor with carbamylcholine as a function of carbamylcholine concentration: (open squares) no added guanyl nucleotide; (filled squares) 50 μM added GppNHp. Error bars indicate scatter of duplicate determinations. Curves represent isotherms calculated from parameter set 4 with values of α_1 and α_2 given in Table I. (A) 5 day old, no pertussis toxin; (B) 5 day old, 25 ng/mL pertussis toxin; (C) 14 day old, no pertussis toxin; (D) 14 day old, 25 ng/mL pertussis toxin.

Table I: Summary of Results of Least-Squares Fitting of Model to Data on Carbamylcholine Binding to Rat Myocyte Membranes

age	PT (ng/mL)	parameter set[a]	α_1^{-b}	α_2^{-b}	α_1^{+b}	α_2^{+b}	RMSE × 10^{2c}	RMSD × 10^{2d}
5	0	1	0.40	0.16	0.02	0.13	3.9	8.5
		2	0.39	0.35	0.01	0.32	3.7	
		3	0.38	0.28	0.00	0.33	3.7	
		4	0.32	0.25	0.00	0.27	3.6	
5	25	1	0.40	0.11	0.60	0.38	5.8	11.1
		2	0.45	0.40	0.63	2.50	5.3	
		3	0.44	0.29	1.00	0.41	6.3	
		4	0.35	0.24	0.60	1.11	5.6	
14	0	1	0.45	0.01	0.63	0.11	3.5	4.6
		2	0.38	0.04	0.61	0.25	3.5	
		3	0.39	0.06	0.64	0.19	3.2	
		4	0.33	0.01	0.88	2.36	2.7	
14	25	1	0.30	0.51	0.25	0.51	4.1	10.4
		2	0.30	0.93	0.26	0.92	4.0	
		3	0.71	0.67	0.96	0.71	4.0	
		4	0.27	0.81	0.24	0.80	4.1	

[a] Parameter sets (set no., K_0, log Q_1, log β_1, log Q_2, log β_2): 1, 4.3, >5, 1.6, >5, −1.05; 2, 4.55, >5, 1.35, 1.2, −3.3; 3, 4.55, 1.2, 1.4, >5, −1.1; 4, 4.5, 1.2, 1.7, 1.2, −3.3. [b] α_1^- and α_2^- are the values of α_1 and α_2 in the absence of added GppNHp; α_1^+ and α_2^+ are the values of α_1 and α_2 in the presence of 50 μM GppNHp. [c] Root square error of fit = $[\sum_i(Y_{i,\text{exp}} - Y_{i,\text{best-fit calc}})^2/\text{dof}]^{1/2}$, where dof (degrees of freedom) = number of points minus number of independently variable parameters (eight per pair of isotherms). [d] Root mean square difference between duplicate determinations = $[\sum_i(Y_i^{(1)} - Y_i^{(2)})^2/(\text{number of points})]^{1/2}$.

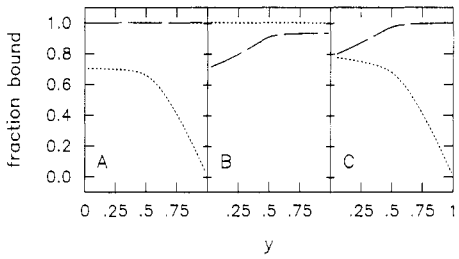


FIGURE 2: Fraction of total X_1 and X_2 bound, plotted as a function of fractional saturation with agonist: (dashed line) X_1; (dotted line) X_2. Panels A–C are calculated from parameter sets 2–4, respectively, with low $K_0 = 4.5$ and $\alpha_1 = \alpha_2 = 0.5$.

does not provide an adequate mechanistic interpretation of the data.

Parameter Set 2: Q_1 *Large*; Q_2 *Moderate* (*Approximately* 10^1). In this case, X_1 binds stoichiometrically to R at all agonist concentrations, and the abundance of the highest affinity class of sites (i.e., RX_1) depends only on the ratio of X_{1t} to R_t (α_1). X_2 binds more weakly to R, and since β_2 is less than unity, agonist competes with X_2 for R. Hence as agonist concentration increases, the amount of RX_2 decreases and X_2 is released from R, as illustrated in Figure 2A.

Parameter Set 3: Q_1 *Moderate*; Q_2 *Large*. In this case, X_2 binds stoichiometrically to R at all agonist concentrations, and the abundance of the lowest affinity class of sites (i.e., RX_2) depends only on the ratio of X_{2t} to R_t (α_2). X_1 binds more weakly to R, and since β_1 is greater than unity, agonist binding enhances the affinity of R for X_1. Hence as agonist concentration increases, the amount of RX_1 increases and unbound X_1 is taken up by R, as illustrated in Figure 2B.

Parameter Set 4: *Both* Q_1 *and* Q_2 *Moderate*. In this case, agonist binding at low agonist concentrations increases the affinity of R for X_1, leading to an uptake of X_1, and at high agonist concentrations decreases the affinity of R for X_2, leading to release of X_2, as illustrated in Figure 2C.

Effect of Batrachotoxin upon Carbamylcholine Binding to Muscarinic Receptors in Rat Atrial Membranes. When batrachotoxin is added to rat atrial membrane preparations, the affinity of muscarinic receptors in these preparations for carbamylcholine is observed first to increase and then subsequently decrease with increasing concentrations of added BTX. It is assumed that when BTX binds to sodium channel, the affinity of sodium channel for X_1 and/or X_2 is altered. The amounts of X_1 and/or X_2 available to interact with muscarinic

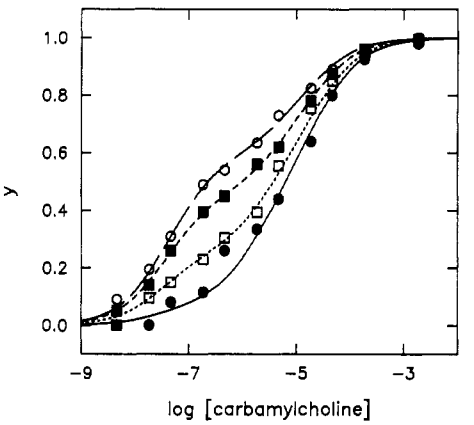


FIGURE 3: Fractional saturation of rat atrial membrane receptor with carbamylcholine as a function of carbamylcholine concentration: (open squares) no added BTX; (filled squares) 0.3 μM BTX; (open circles) 1.0 μM BTX; (filled circles) 3.0 μM BTX. Curves represent isotherms calculated from parameter set 3a with values of α_1 and α_2 given in Table II.

receptors (quantified in the model as α_1 and α_2) change correspondingly, and the affinity of muscarinic receptor for carbamylcholine is thereby modified.[5] The model introduced above was simultaneously fit to replicate data on the binding of carbamylcholine to muscarinic receptors in the absence of and in the presence of three concentrations of BTX. The values of K_0, Q_1, Q_2, β_1, and β_2 were constrained to be the same for all concentrations of BTX, as BTX is not expected to influence either the affinities of R, RX_1, and RX_2 for carbamyl choline or the affinities of X_1 and X_2 for R.

We found that these data sets could be satisfactorily fit with (at least) four different sets of values of K_0, β_1, β_2, Q_1, and Q_2, each of which is similar to or identical with one of the corresponding sets of parameter value sets used to fit the carbamylcholine binding data obtained from cultured rat

[5] The assumption that the effect of BTX upon agonist binding to muscarinic receptors can be adequately parameterized in terms of alterations in α_1 and α_2 is clearly an oversimplification. A more realistic model would have to take into account the equilibrium nature of the binding of X_1 and/or X_2 to BTX-linked sites on the sodium channel. However, currently available data on the binding of BTX to sodium channels in the presence of varying amounts of muscarinic agonists and antagonists (Cohen-Armon & Sokolovsky, 1986) are too sparse to permit a more complete quantitative treatment.

Table II: Summary of Results of Least-Squares Fitting of Model to Data on Carbamylcholine Binding to Rat Atrial Membranes[a]

parameter set[b]		BTX (μM)	α_1	α_2	RMSE × 10^2[c]
1	α_2 variable	0.00	0.24	0.33	1.6
		0.3	0.44	0.31	
		1.0	0.55	0.23	
		3.0	0.11	0.46	
	α_2 constant	0.0	0.28	0.14	2.0
		0.3	0.46	0.14	
		1.0	0.59	0.14	
		3.0	0.12	0.14	
2	α_2 variable	0.0	0.25	0.74	1.6
		0.3	0.46	0.81	
		1.0	0.57	0.75	
		3.0	0.10	0.87	
	α_2 constant	0.0	0.27	0.78	1.7
		0.3	0.47	0.78	
		1.0	0.59	0.78	
		3.0	0.10	0.78	
3	α_2 variable	0.0	0.25	0.48	1.8
		0.3	0.46	0.41	
		1.0	0.56	0.30	
		3.0	0.14	0.63	
3a	α_2 constant	0.0	0.27	0.15	2.0
		0.3	0.46	0.15	
		1.0	0.59	0.15	
		3.0	0.12	0.15	
4	α_2 variable	0.0	0.23	0.62	1.7
		0.3	0.48	0.78	
		1.0	0.59	0.67	
		3.0	0.07	0.72	
	α_2 constant	0.0	0.25	0.70	1.7
		0.3	0.46	0.70	
		1.0	0.59	0.70	
		3.0	0.12	0.70	

[a] Root mean square difference between duplicate determinations = 2.2×10^{-2}. [b] Parameter sets (set no., log K_0, log Q_1, log Q_2, β_1, β_2): 1, 5.6, >5, >5, 1.9, −1.05; 2, 6.0, >5, 1.35, 1.35, −3.3; 3, 5.8, 1.2, >5, 1.7, −1.1; 3a, 5.2, 1.2, >5, 2.2, −1.1; 4, 5.9, 1.2, 1.2, 1.7, −3.3. [c] Root mean square error of best fit = $[\sum_i (Y_{i,exp} - Y_{i,best\text{-}fit\ calc})^2/dof]^{1-2}$, where dof (degress of freedom) = 31 for α_2 variable and 34 for α_2 constant.

myocytes. These parameter sets are presented in Table II, and a representative set of calculated isotherms is plotted together with the data in Figure 3. Isotherms calculated with other sets of parameter values shown in Table II are not plotted, as they are almost indistinguishable from those shown.

It was subsequently found that the data could be fit with a more restricted model, wherein the total amount of X_2 interacting with receptor is constrained to be independent of BTX concentration. Once again, four sets of parameter values were explored and found to provide a fit of the model to the data within experimental precision. These parameter values are presented in Table II. As in the case of agonist binding to rat myocyte membranes, even though the set of parameter values with indefinitely high values of Q_1 and Q_2 (i.e., the fixed three-site-class model) provides an acceptable fit to the carbamylcholine binding data, it cannot account for the effect of muscarinic agonists on the affinity of sodium channel for BTX (Cohen-Armon & Sokolovsky, 1986) and is hence incompatible with our expectation that agonist binding is linked to the uptake or release of a second component.

Finally, we attempted to fit the combined data with a second constrained model, in which X_1 was assumed to be independent of BTX concentration and X_2 was allowed to vary with BTX concentration. We were unable to find a single set of parameter values that would provide an acceptable fit.

Discussion

Within the context of the extended ternary complex model presented here there are a variety of physical possibilities consistent with the binding isotherms shown in Figures 1 and 3. These include (but are not limited to) (a) the uptake of X_1 associated with high-affinity binding of agonist, (b) the release of X_2 associated with low-affinity binding of agonist, and (c) a combination of possibilities a and b. We cannot rank these possibilities in order of preference on the basis of the binding data alone. However, until such time as new data require us to simultaneously take into account both agonist-linked uptake of X_1 and release of X_2, we will limit our analysis to the simpler alternatives a and b.

We are able to fit the combined data obtained at various concentrations of BTX to a model in which only X_1 varies with BTX concentration, suggesting that the binding of BTX to sodium channel may be coupled to release and uptake of only a single allosteric effector (X_1). This simplified model can also account for the independent observation that BTX binding (at BTX concentrations of up to 1 μM) is enhanced by the presence of carbamylcholine if we postulate that the binding of BTX to the highest affinity binding site on sodium channel is linked to a release of X_1. The observation that at high BTX concentrations the affinity of muscarinic receptor for carbamylcholine is decreased suggests that binding of BTX to lower affinity sites on sodium channel is linked to an uptake of X_1.

It has been reported that addition of GppNHp inhibits the enhancement of BTX binding otherwise observed in the presence of muscarinic agonist (Cohen-Armon & Sokolovsky, 1986). The ETC model provides a straightforward rationalization of this effect. The observation that the addition of GppNHp to preparations of rat atrial membranes lowers the overall affinity of receptor for muscarinic agonist (Gurwitz et al., 1985) implies (within the context of the model) that GppNHp reduces or eliminates the interaction between receptor and that species, namely, X_1, which increases the affinity of receptor for agonist. It follows that addition of GppNHp will reduce or eliminate the linkage between agonist binding to muscarinic receptor and the size of the pool of X_1 that is available to modulate the binding of BTX to sodium channel.

The simplest model rationalizing both the observed effects of BTX on carbamoylcholine binding and of carbamylcholine on BTX binding postulates that both processes are linked to uptake and/or release of a single additional component (X_1). That set of parameters used to fit the carbamylcholine binding data, which has an indefinitely large value of Q_1, cannot account for agonist-linked uptake of X_1 and is thus incompatible with this simple model. A more complex model in which both X_1 is taken up and X_2 is released as muscarinic receptors are saturated with agonist is, however, equally compatible with the data.

We emphasize that even in the case where agonist is not linked to the release of X_2, other chemical changes in the cell that increase or decrease the amount of X_2 that can bind to muscarinic receptors will modulate the agonist binding properties of these receptors.

A qualitative summary of the effects of addition of GppNHp and or pertussis toxin upon the best-fit values of total X_1 and X_2 in young and old myocyte membranes is presented in Table III. The present model postulates that the affinities of individual receptor substates R, RX_1, and RX_2 for agonist are unaffeced by age. According to this model, the age dependence of agonist binding is attributable to variations in the amounts of substances such as X_1 and X_2 that can bind to and alter the affinity of receptor for agonist. Similarly, changes in agonist binding associated with the addition of GppNHp and/or pertussis toxin are attributed not to alterations in the

Table III: Effects of Added GppNHp and Pertussis Toxin on Best-Fit Values of α_1 and α_2 in Ternary Complex Model

	GppNHp	PT	GppNHp + PT
young myocytes (5 day)	$\alpha_1 \downarrow$		$\alpha_1 \uparrow$ $\alpha_2 \uparrow$
aged myocytes (14 day)	$\alpha_1 \uparrow$ $\alpha_2 \uparrow$	$\alpha_2 \uparrow$	$\alpha_2 \uparrow$

affinity for agonist of R, RX_1, or RX_2 but to the effect of GppNHp and/or pertussis toxin on the amount of X_1 and/or X_2 that can interact with receptor. Although both GppNHp and PT bind strongly and quasi-irreversibly to G-proteins, presumably altering the ability of the substrate G-proteins to interact with muscarinic and other receptors, the effects of the two substances do not appear to be additive. Age-related changes in the lipid content of myocyte membranes [see Moscona-Amir et al. (1986, 1989) and references cited therein] can in principle affect the amounts of X_1 and/or X_2 that are accessible to and can interact with muscarinic receptor, simply by altering the distribution of intrinsic proteins within and between various microdomains. It is possibly relevant to note that the accessibility of pertussis toxin substrates in this system (including Gi-protein) to exogenous pertussis toxin has been reported to be age dependent (Moscona-Amir et al., 1989).

In fitting the extended ternary complex model to data on the binding of carbamylcholine to rat myocyte membranes under a variety of conditions, we have postulated that the effects upon the agonist binding isotherm of varying the concentrations of GppNHp and PT and the age of a myocyte may be taken into account by altering the amount of X_1 and/or X_2 that is competent to interact with receptor, without perturbing the strength of the interaction between receptor and effector. While this physical picture is very likely an oversimplification, the parameterization of what may be a variety of complex changes in the system as simple alterations in α_1 and α_2 is permissible from a formal point of view. The reason for this is that one may formally consider any complex of X_1 and an additive such as GppNHp, pertussis toxin, or an unknown age-dependent substance to constitute yet another species of X: for example, we could have defined the hypothetical reactions $X_1 + PT \rightarrow X_3$ or $X_4 + GppNHp \rightarrow X_2$. If the species X_3 and X_4 do not interact with R (that is to say, Q_3 and $Q_4 = 0$), then these species will not explicitly appear in the reaction scheme or equations describing our model. However, the end result is that, in the case of the first hypothetical reaction, the addition of PT to the preparation results in a decrease in total X_1, parameterized as a decrease in α_1, and in the case of the second hypothetical reaction, the addition of GppNHp results in an increase in total X_2, parameterized as an increase in α_2. We stress that no claim is made that only X_1 and X_2 interact with R. However, we do not need to postulate agonist-dependent interactions with more than two varieties of X in order to accommodate any agonist-binding data of which we are aware.

We have shown that the data presented here can be fit by a model in which the formation of a complex with one effector, X_1, increases the affinity of receptor for agonist and the formation of a complex with a second effector, X_2, decreases the affinity of receptor for agonist. However, the values of the model parameters are not uniquely determined by the data. For example, we have obtained preliminary results suggesting that the data may also be fit with an extended ternary model in which the lowest affinity species is uncomplexed R and both RX_1 and RX_2 have higher affinities for agonist or a model in which the highest affinity species is uncomplexed R and both RX_1 and RX_2 have lower affinities for agonist. Thus, we caution the reader against attributing significance to the particular values of the parameters presented here. We stress that the significance of the work presented here is qualitative rather than quantitative:

(1) We have established that a variety of data on the binding of agonists to muscarinic receptors, heretofore regarded as incompatible with a ternary complex model, are in fact well accommodated by a straightforward extension of that model. In particular, the extended ternary complex model provides a reasonable explanation of the heretofore puzzling observation that addition of GppNHp increases agonist affinity in aged myocyte cultures while decreases agonist affinity in young myocyte cultures.

(2) We have established that an extended ternary complex model can in principle account for both the effect of BTX upon the binding of agonists to muscarinic receptors and the reciprocal effect of muscarinic agonists upon the binding of BTX to sodium channels. The present quantitative formulation is entirely consistent with a previously proposed qualitative picture of "cross talk" between distinct classes of signal-transducing moieties in membranes (Sokolovsky et al., 1986; Sokolovsky, 1989).

(3) The model parameters Q_1 and Q_2 are measures of the strength of the interaction between receptor and each of the effector substances and are therefore expected to be independent of the particular agonist employed in a given experiment. The parameters β_1 and β_2, on the other hand, are measures of the respective affinities of RX_1 and RX_2 for agonist relative to that of R. These parameters could reasonably be expected to vary with the particular nature of the agonist. Hence, the model provides a straightforward qualitative explanation of why agonists exhibiting different binding isotherms (such as carbamylcholine and oxotremorine) interact differently with other cellular components (Cohen-Armon & Sokolovsky, 1986).

A substantial number of G-proteins are now known to interact with muscarinic receptors [for a review, see Sokolovsky (1989)]. While G-proteins are obvious candidates for the role of the effector substances X_1 and X_2, other cellular components should not be excluded in the absence of direct biochemical evidence. Moreover, each of the X_i may represent not just a single molecular species but an entire class of substances that interact similarly with muscarinic receptor. The analysis presented here is not meant to imply that only two effector substances (or classes of effector substances) can modify the agonist-binding properties of these receptors or that agonist binding is linked to uptake and/or release of only two effector substances. Rather, it is meant to show quantitatively that *no less than* two ternary complexes are required in order to accommodate the data that have so far been obtained.

Appendix

Numerical Solution of Model Equations. We define the following scaled concentrations:

$$[L'] \equiv K_0[L] \qquad [R'] \equiv [R]/R_t$$
$$[X_1'] \equiv [X_1]/R_t \qquad [X_2'] \equiv [X_2]/R_t \tag{A1}$$

Using these concentrations together with the scaled parameters defined in eq 6, eqs 2–4 may be rewritten:

$$1 = [R']\{(1 + [L']) + Q_1[X_1'](1 + \beta_1[L']) + Q_2[X_2'] \times (1 + \beta_2[L'])\} \tag{2'}$$

$$\alpha_1 = [X_1']\{1 + Q_1[R'](1 + \beta_1[L'])\} \tag{3'}$$

$$\alpha_2 = [X_2']\{1 + Q_2[R'](1 + \beta_2[L'])\} \tag{4'}$$

Equations 3′ and 4′ may be rearranged to yield expressions

for $[X_1']$ and $[X_2']$, respectively. When these are substituted into eq 2, one obtains

$$[R'] = 1/\left\{\phi_0 + \frac{\phi_1\alpha_1}{1 + \phi_1[R']} + \frac{\phi_2\alpha_2}{1 + \phi_2[R']}\right\} \quad (A2)$$

where

$$\phi_0 = 1 + [L']$$
$$\phi_1 = Q_1(1 + \beta_1[L'])$$
$$\phi_2 = Q_2(1 + \beta_2[L'])$$

Equation A2 may be solved iteratively to yield the value of $[R']$. Upon substitution of this value into eqs 3′ and 4′, the values of $[X_1']$ and $[X_2']$ are obtained. Then, the fractional saturation of agonist is calculated via the following transformation of eq 5:

$$y = [L'][R'](1 + Q_1\beta_1[X_1'] + Q_2\beta_2[X_2']) \quad (5')$$

Registry No. GppNHp, 34273-04-6; BTX, 23509-16-2; carbamylcholine, 462-58-8.

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Mechanism of Spontaneous, Concentration-Dependent Phospholipid Transfer between Bilayers†

Jeffrey D. Jones and T. E. Thompson*

Department of Biochemistry, University of Virginia, Charlottesville, Virginia 22908

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ABSTRACT: We have previously demonstrated that spontaneous phospholipid transfer between bilayer vesicles at higher vesicle concentrations is characterized not only by a first-order desorption rate but also by a second-order process dependent on vesicle concentration (Jones & Thompson, 1989b). We have extended our studies to examine the mechanism of this second-order process by investigating transfer as a function of lipid type, temperature, aqueous medium composition, and vesicle size. The results suggest a mechanism of concentration-dependent transfer in which the rate of lipid monomer desorption from vesicle bilayers is enhanced in transient vesicle–vesicle complexes.

The fact that lipids spontaneously transfer between biological and bilayer membranes in aqueous dispersion is well established [for reviews, see Sleight, (1987); Dawidowicz, (1987); and Phillips et al. (1987)]. It is generally recognized that transfer is independent of vesicle concentration in the low vesicle concentration range at which earlier studies have been

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