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Regulation of binding properties of the nicotinic receptor protein by cholinergic ligands in membrane fragments from *Torpedo marmorata*

(structural transition/pharmacological desensitization/regulatory protein)

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ABSTRACT **Exposure of receptor-rich membrane fragments from *Torpedo marmorata* to carbamylcholine causes a slow (half-time of 5–10 min) and reversible change of properties of the cholinergic receptor protein manifested by a decrease of the initial rate of *Naja nigricollis* α-[^{3}H]toxin binding in the presence of carbamylcholine. This change corresponds to a 5- to 20-fold increase of affinity for carbamylcholine. Other agonists, acetylcholine, phenyltrimethylammonium, show the same effect but not the antagonists *d*-tubocurarine and flaxedil. Decamethonium and hexamethonium show little, if any, agonistic effect *in vitro* on the same membrane fragments but cause the affinity change. This regulatory property can be lost after aging of the preparation of membrane fragments. Since the affinity increase progresses with a similar time course as the decrease of amplitude of the permeability response consecutive to agonist preincubation, it is proposed that, in the membrane at rest, the receptor protein is present under a state of low affinity for agonists and that the reversible stabilization by the agonists of a high affinity state corresponds to the "pharmacological desensitization" of the system as predicted by one of the models of Katz and Thesleff.**

Examination of the values reported in the literature for the intrinsic dissociation constants between cholinergic ligands, mainly agonists, and the cholinergic receptor protein is a rather bewildering experience. With acetylcholine, for instance, these values range between 10^{-8} M (high affinity) and 10^{-5} M (low affinity). Early reports on the receptor protein from *Electrophorus* either in Na deoxycholate crude extracts (1, 2) or in purified membrane fragments (3, 4) gave low affinities for agonists; but after purification of the receptor protein on an affinity column (with a cholinergic molecule attached) in the presence of Triton X-100, the protein exhibited a 15–40 times higher affinity for the same agonists (5). Receptor-rich membrane fragments from *Torpedo* bind acetylcholine essentially with a single high affinity (4), whereas the receptor protein purified in the presence of Triton X-100 possesses either a low affinity for agonists (cholinergic column) (6) or a mixture of low and high affinities (α-toxin column) (7). That a fundamental microheterogeneity of the binding constants exists for agonists (8) has been confirmed from studies on dissolution by cholate of receptor-rich membrane fragments (8, 9, 10). Finally, it was shown that after Na cholate dissolution, an interconversion between high and low affinity states takes place upon variation of the concentration of detergent (10). The cholinergic receptor protein may therefore exist, at least in solution, in several interconvertible states of affinity.

The functional significance of these multiple states of affinity can be challenged by experiments with the receptor-rich membrane fragments from *Torpedo*, since they still respond, *in vitro*, to the agonists by an increase of $^{22}Na^+$ permeability (11). Interestingly, the *apparent* dissociation constants determined under these conditions from the dose-response curves fall, as in the case of *Electrophorus*, in a range very close to those of the low affinity state (12). Would then the high affinity state be a nonfunctional conformation of the receptor?

New insights to this question were reached in the observation that upon prolonged exposure *in vitro* of the receptor-rich microsacs to agonists, the amplitude of the permeability response decreases in a slow and reversible manner. As in the case of the neuromuscular junction (13) or the live electroplaque (14), pharmacological "desensitization" takes place. Several models have been proposed to account for the phenomenon of desensitization; one of them (13) postulates that in the "desensitized" state, in which the ionophore is shut, the receptor exhibits a high affinity for agonists. The finding that local anesthetics or calcium ions, known to enhance desensitization *in vivo* (15) and *in vitro* (12), do stabilize the receptor protein in a high affinity state in *Torpedo* membrane fragments (9) brought experimental evidence in favor of this interpretation.

In this communication, we report on experiments done with receptor-rich membranes from *Torpedo marmorata*. The affinity of the receptor site for cholinergic ligands was measured, indirectly, by following the decrease of the initial rate of *Naja nigricollis* α-[^{3}H]toxin binding (4). The experiments show that, in the membrane, the receptor protein is spontaneously present in a state which binds cholinergic agonists with a low (or medium) affinity and that cholinergic agonists, themselves, stabilize in a slow and reversible manner the receptor protein in a high affinity state for agonists.

MATERIAL AND METHODS

The tritiated α-neurotoxin from *Naja nigricollis* was labeled by the method of Menez *et al.* (16) and was a gift of Drs. Boquet, Menez, Morgat, and Fromageot. The specific activity of the batch used was 15 Ci/mmol and 65% of the α-toxin molecules were considered as pharmacologically active (4) at the time of the experiment. All toxin concentrations are expressed in terms of *active* α-toxin molecules.

Receptor-rich membrane fragments were prepared from fresh electric tissue of *Torpedo marmorata* by the method of Cohen *et al.* (17) as modified by Hazelbauer and Changeux (11). The final pellet was resuspended in 1.2 M sucrose, 0.02% NaN_3 with a Potter glass-Teflon homogenizer and stored at 0°. The specific activity of the membrane preparations ranged between 500 and 1000 nmol of α-[^{3}H]toxin sites per gram of protein.

Abbreviations: Carb, carbamylcholine; PTA, phenyltrimethylammonium.

Binding of α-[^{3}H]toxin to the receptor-rich membrane fragments was followed by filtration on Millipore (HAWP 02500) as described by Weber and Changeux (4). The reaction mixture (15 ml) contained: (*1*) 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Na phosphate, pH 7.0 (*Torpedo* physiological solution); (*2*) 0.5 nM α-[^{3}H]toxin; (*3*) from 3 to 5 nM α-[^{3}H]toxin binding sites; (*4*) the cholinergic effector tested. In the case of acetylcholine, acetylcholinesterase was inhibited by incubating the membrane fragments for at least 2 hr at 4° with 0.1 mM Tetram (8). Binding of the α-toxin was then followed in the presence of 0.01 mM Tetram. Under these conditions, the classical semi-logarithmic plot for a pseudo first-order reaction gave a straight line for at least 15 min, which corresponds to the occupancy of at least 70% of the α-toxin sites.

The reaction was started by mixing toxin and membrane fragments. At given times, 0.5- or 2-ml samples were taken, filtered, and washed on the filter with 10–15 ml of physiological solution. The dried filters were counted in an Intertechnique SL30 scintillation counter. The number of cpm given by extrapolation of the binding kinetics at zero time was systematically subtracted from these data; whenever checked this value was the same as the radioactivity of the filter after filtration of the same quantity of α-[^{3}H]toxin solution but in the absence of membrane fragments. The variation, observed occasionally, in this background was due either to changes of water pressure during the filtration and/or to differences between batches of filters.

RESULTS

(1) Time-dependent modification of the cholinergic receptor protein by carbamylcholine

In previous binding studies with receptor-rich membrane fragments from *Torpedo* (4) the dissociation constants for cholinergic ligands were measured either directly by centrifugation with radioactive cholinergic effectors or by following the decrease of the initial rate of α-[^{3}H]toxin binding as a function of cholinergic ligand concentration. Both methods gave concordant high affinities for agonists. In all these experiments, however, and on purpose, the membrane fragments were equilibrated with the cholinergic effectors for at least 10 min (most often 1 hr) prior to centrifugation or addition of α-[^{3}H]toxin. The possibility became soon evident that, during such a prolonged exposure, the binding properties of the membrane bound receptor protein might change. To test this possibility, toxin binding measurements were carried out to shorten the time of sampling, as well as the time of contact of the membrane fragments with the cholinergic ligands. For this purpose, the membrane suspension was added to a pre-established mixture of α-[^{3}H]toxin and cholinergic ligands ("zero minutes incubation" in Fig. 1).

Fig. 1 (left) shows the kinetics of α-[^{3}H]toxin binding performed under these conditions. As expected (4), in the absence of carbamylcholine the reaction progresses linearly with time for at least 4.5 min; but, in the presence of 0.25 μM carbamylcholine, after an initial start with the same rate as the control, the reaction slows down 2 min later; in the presence of 2.5 μM carbamylcholine the rate initially decreases by approx. 40% and becomes negligible after 2.5 min. These results differ from those we previously observed when the toxin was added to an equilibrated mixture of carbamylcholine and membrane fragments (4): in all circumstances, the kinetics were linear and the decrease of the initial rates by carbamylcholine was more pronounced. Preincubation of the membrane fragments with carbamylcholine, therefore, modifies the properties of the system.

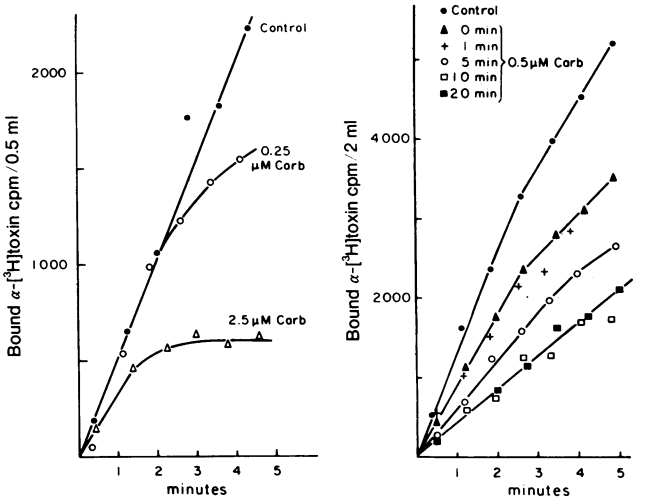

FIG. 1. (*Left*) Effects of carbamylcholine (Carb) on the kinetics of α-[^{3}H]toxin binding to *Torpedo* membrane fragments in the absence of preincubation. Membrane fragments are diluted to a final concentration of 5.0 nM α-toxin binding sites in *Torpedo* physiological solution supplemented with 0.8 nM α-[^{3}H]toxin (22 Ci/mmol) and the indicated concentration of carbamylcholine. Samples (500 μl) are filtered at the indicated times. Initial rate of the control kinetics: 0.15 nM min^{-1}. (*Right*) Effect of the time of preincubation of membrane fragments with carbamylcholine on the kinetics of α-[^{3}H]toxin binding. Concentration of α-[^{3}H]toxin: 0.5 nM; of toxin binding sites: 3.6 nM. The membrane fragments were incubated for the indicated time in 15 ml of *Torpedo* physiological solution supplemented with 0.5 μM carbamylcholine before the reaction was started by adding α-[^{3}H]toxin. 0 min preincubation was obtained by diluting the membrane fragments in the mixture of α-[^{3}H]toxin and carbamylcholine. 60 min incubation with carbamylcholine resulted in a kinetics superimposable with that obtained with 10 or 20 min (not shown). Initial rate of control kinetics: 0.06 nM min^{-1}.

The experiment illustrated by Fig. 1 (right) gives further credence to this conclusion. Membrane fragments were first exposed for increasing lengths of time with 0.5 μM carbamylcholine, and then the toxin was added. The initial rate of toxin binding decreases with the length of the preincubation period. After 10–20 min, it reaches a minimal value, characteristic of the concentration of carbamylcholine present, and no longer varies with the time of preincubation. This maximal inhibition of the initial rate of toxin binding is identical to that found in our previous studies at the same concentration of carbamylcholine (4). Control experiments carried out in *Torpedo* physiological solution but in absence of carbamylcholine confirm the fact that carbamylcholine by itself causes a time-dependent modification of the membrane-bound receptor. The time course of the modification varies from one membrane preparation to another, with times for half modification ranging from 5 to 10 min.

The reversibility of the observed modification was tested by diluting 75 times a suspension of membrane fragments previously exposed to 0.5 μM carbamylcholine for 20 min (Fig. 2). To check first that the modification had occurred, a sample was diluted directly in a mixture of α-[^{3}H]toxin and 0.3 μM carbamylcholine (Fig. 2, "0 min recovery"); as expected, the initial rate of α-[^{3}H]toxin was 50% that observed in the complete absence of carbamylcholine.

Membrane fragments preincubated with 0.5 μM carbamylcholine were then diluted in *Torpedo* physiological solution. Five or 10 min after dilution (recovery time), carbam-

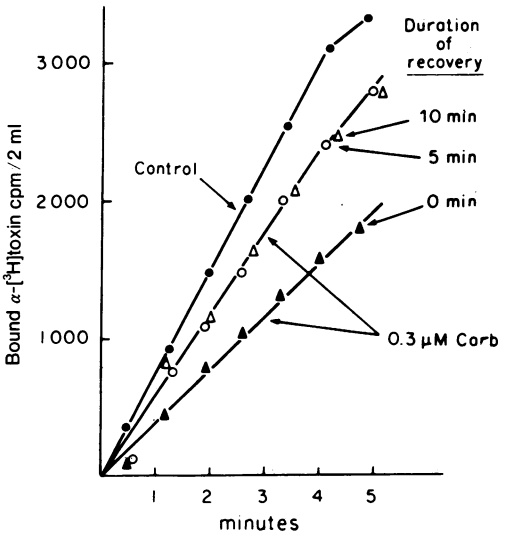

FIG. 2. Reversibility of the affinity change caused by preincubation of membrane fragments with carbamylcholine. Ninety microliters of a membrane suspension in 1.2 M sucrose (9.0 μM α-toxin binding sites) were diluted to 300 μl with *Torpedo* physiological solution and supplemented with 0.5 μM carbamylcholine. After 10 min incubation at 20°, 200 μl were diluted in 15 ml of physiological solution: the final concentration of toxin binding sites was then 3.6 nM, that of carbamylcholine, 6.7 nM. After 5 or 10 min, the reaction was started by adding simultaneously 0.5 nM α-[³H]toxin and 0.3 μM carbamylcholine (final concentrations). "0 min recovery" means that 200 μl of preincubation medium were diluted in 15 ml of *Torpedo* physiological solution supplemented with 0.5 nM α-[³H]toxin and 0.3 μM carbamylcholine. The control kinetics was measured when neither the preincubation medium nor the reaction medium contained carbamylcholine. Diluting 200 μl of the preincubated mixture containing 0.5 μM carbamylcholine in 15 ml of 0.5 nM α-[³H]toxin without carbamylcholine resulted in a kinetics exactly superimposable with the control (not shown). Initial rate of control kinetics: 0.045 nM min^{-1}.

ylcholine (final concentration 0.3 μM) and α-[³H]toxin were added simultaneously. Under these conditions, the initial rate of toxin binding was reduced by only 20% (Fig. 2). This rate becomes identical to that observed when the membrane fragments were *not* preincubated with 0.5 μM carbamylcholine, and directly diluted in the mixture of α-[³H]toxin and 0.3 μM carbamylcholine (not shown). The modification of the receptor protein caused by carbamylcholine is thus entirely reversible.

The time course of the recovery was studied under the same conditions with another preparation of membrane fragments. At the time of dilution and 15 sec, 1 and 5 min later, a mixture of α-[³H]toxin and carbamylcholine (final concentration 0.3 μM) was added. A 10% recovery took place 15 sec. after dilution, 35% after 1 min, and 70% after 5 min.

In order to test the possibility that the slowly reversible modification could be due to a change in the bimolecular rate constant of toxin binding, an experiment identical to that described above was carried out but in which α-[³H]toxin alone was added, instead of a mixture of α-[³H]toxin and carbamylcholine. Any time after dilution, the kinetics of toxin binding was identical to that observed with membrane fragments which were *never* exposed to carbamylcholine. In other words, preincubation with carbamylcholine does not significantly affect the intrinsic rate of association of the α-toxin with the receptor protein. Therefore, the time-dependent modification of the receptor protein corresponds to an increase of affinity for carbamylcholine.

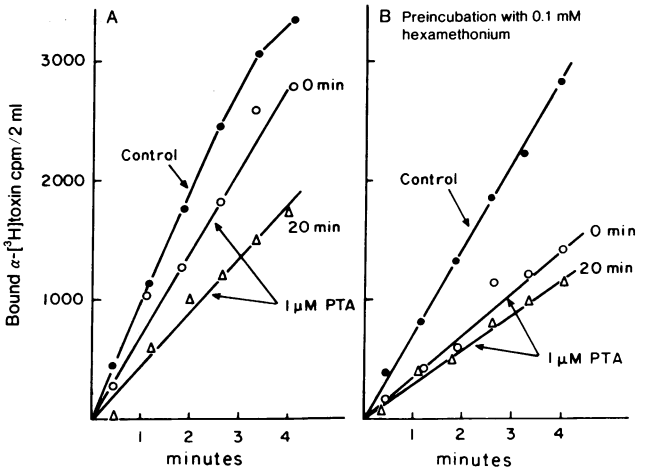

FIG. 3. Effect of a preincubation of membrane fragments with hexamethonium on the affinity of the receptor for phenyltrimethylammonium. (A) 100 μl of membrane fragments (0.7 μM toxin binding sites) were diluted in 500 μl of *Torpedo* physiological solution (conditioning medium). The kinetics of α-[³H]toxin binding after 0 min and 20 min incubation in the presence of 1 μM PTA was then measured as described in the legend of Fig. 1. Final concentrations of toxin binding sites: 3.9 nM; of α-[³H]toxin: 0.5 nM. Initial rate of control: 0.046 nM min^{-1}. (B) The same experiment as in A, but the conditioning medium was supplemented with 0.1 mM hexamethonium. The final concentration of hexamethonium in the reaction medium was 3.3 μM.

(2) Pharmacological specificity of the change of affinity

Experiments similar to those described with carbamylcholine have been repeated with decamethonium, phenyltrimethylammonium, and acetylcholine (in the presence of 10^{-5} M Tetram). With each one of these ligands, preincubation enhances the protective effects against α-toxin binding (Table 1). On the other hand, the typical antagonists *d*-tubocurarine or gallamine triethiodide (flaxedil) do not produce this effect. In the case of hexamethonium, the situation appears less clear. After a preincubation of 20 min, the kinetics of α-toxin binding grossly parallels that observed in the 0 min preincubation sample but often is slightly displaced towards higher concentration of bound toxin.

The structural specificity of the change of affinity associated with preincubation with a given cholinergic ligand was investigated in a two-step experiment. The membrane fragments in a small volume of physiological solution were first exposed to a "conditioning" concentration of effector. Then, the mixture was diluted approximately 100 times in a solution of a second effector, the concentration of the first one becoming negligible. After 0 and 20 min incubation with the second ligand, rates of α-toxin binding were measured. Table 2 shows the results of a series of such experiments. Preincubation with 0.3 μM carbamylcholine clearly enhances the affinity for another agonist, phenyltrimethylammonium, but not that for an antagonist *d*-tubocurarine (not shown). On the other hand, preincubation with the antagonist gallamine results only in a slight, if any, increase of affinity of the receptor protein for phenyltrimethylammonium and decamethonium.

An interesting situation is offered by hexamethonium which, as already mentioned, seems to exhibit only a small preincubation effect as far as its own affinity is concerned. However, as shown in Fig. 3, preincubation of the membrane fragments with 0.1 mM hexamethonium for 20 min markedly increases the affinity for phenyltrimethylammon-

Table 1. Effect of cholinergic ligands on the initial rate of α-[^{3}H]toxin binding with and without preincubation of the membrane fragments with the same ligands

	0.4 μM Carb	20 nM ACh	80 μM PTA	1 μM Deca	80 μM Hexa	11 μM Gallamine	0.3 μM *d*-tubo
V 0 min	71	62	76	78	48	34	49
V 20 min	49	30	50	46	42	34	46
$100 \times \frac{V_0 - V_{20}}{V_{20}}$	45	106	52	69	14	0	7

The results in the first two lines are expressed as a percentage of the initial rate measured in the absence of ligand. Carb, carbamylcholine; ACh, acetylcholine; PTA, phenyltrimethylammonium; Deca, decamethonium; Hexa, hexamethonium; *d*-tubo, *d*-tubocurarine.

ium up to the same extent as phenyltrimethylammonium itself.

All these results suggest that the change of affinity once triggered is independent of the particular structure of the triggering ligand, in other words, it takes place between discrete states of the receptor protein. This interpretation is supported by the observation that such transition might occur in the absence of cholinergic ligands.

(3) Spontaneous changes of properties of the membrane-bound receptor in the absence of cholinergic ligands

Storage of membrane fragments at 4° in 1.2 M sucrose and 0.02% NaN_3 is, generally, accompanied by an alteration of the properties of the membrane-bound receptor. As shown in Table 3, the amplitude of the preincubation effect of carbamylcholine decreases after 8 days. It disappears completely after 14 days, a time when the receptor protein reacts with cholinergic ligands and α-toxin as if it was stabilized in its high affinity state. This spontaneous modification affects the binding of carbamylcholine and acetylcholine but not that of antagonists. As expected from our previous discussion, no significant alteration of the initial rate of toxin binding was ever noticed as a consequence of the affinity change for agonists. Because of the rather long mixing time (in the order of 15 sec), it cannot yet be decided whether storage spontaneously stabilizes the receptor in its high affinity state before it is to interact with cholinergic ligands or if, for instance, storage enhances the rate of the affinity increase; the conversion to the high affinity state would then take place within the mixing time lapse.

The time course of the spontaneous change of property of the receptor observed upon storage varies from one membrane preparation to the other from 2 to 14 days. In three of the eleven preparations studied, the cholinergic receptor protein reacted as if it was in its high affinity state 1 day after the membranes were prepared.

Table 2. Effect of preincubation of the membrane fragments with gallamine triethiodide or carbamylcholine on the affinity of the membrane bound receptor protein for phenyltrimethylammonium or decamethonium

	Conditioning		
Test	Preincubation in physiological solution	Preincubation with 8 μM gallamine (20 min)	Preincubation with 0.3 μM Carb (20 min)
Control	100	107	96
1 μM PTA 0 min	65	63	36
1 μM PTA 20 min	31	27	32
Control	100	94	
1 μM Deca 0 min	82	63	
1 μM Deca 20 min	44	44	

The conditions are exactly the same as for Fig. 3. Abbreviations are as in Table 1. Results are expressed as percentage of the control kinetics

DISCUSSION

The prolonged contact of *T. marmorata* receptor-rich membrane fragments with carbamylcholine causes a slow and reversible change of the properties of the membrane-bound receptor as observed by a decrease of the initial rate of toxin binding measured in the presence of carbamylcholine.

At least two different categories of explanations might account for this phenomenon: (*1*) exposure to carbamylcholine causes a change in the intrinsic rate of association of the α-toxin with the receptor protein without significantly altering the affinity for carbamylcholine; (2) preincubation of the receptor protein with carbamylcholine modifies the affinity of the receptor site for carbamylcholine but shows less significant effect on the rate of association of the toxin with the free receptor sites.

If the first hypothesis were correct, then, in the recovery experiment (Fig. 2) the rate of α-toxin binding measured immediately after dilution *in the absence* of carbamylcholine would be smaller than in the nonpreincubated control. In fact, this does not happen.

In a previous work (4), it was shown that after extensive equilibration with cholinergic ligands the fractional decrease of the initial rate of α-toxin binding observed in the pres-

Table 3. Spontaneous change of properties of the membrane-bound receptor protein during storage of the membrane fragments

	% of initial rate in the presence of 0.26 μM carbamylcholine	
Duration of storage, days	0 min incubation, V_0	10 min incubation, V_{10}
1	83	40
8	76	52
14	50	54

Membrane fragments were stored at 0° in 1.2 M sucrose, 0.02% NaN_3. The decrease of the initial rate of α-[^{3}H]toxin binding with and without preincubation with 0.26 μM carbamylcholine was measured as described in legend of Fig. 1 (right). Results are expressed as a percentage of the control kinetics.

ence of a given concentration of ligand coincides with the occupancy of the receptor sites by this ligand (measured directly with tritiated acetylcholine or decamethonium). According to the first hypothesis, the occupancy of the sites by carbamylcholine should not vary during the transition and given concentrations of carbamylcholine should affect to the same extent the rates of toxin binding at the beginning of the incubation period and at its end. Fig. 1 clearly shows that this is not the case. The hypothesis of a time-dependent change of affinity for carbamylcholine seems therefore the most plausible one; the initial rate measurements presently available are compatible with an increase of affinity for carbamylcholine in the range of 5- to 20-fold consecutive to preincubation.

Acetylcholine and phenyltrimethylammonium show the same effect as carbamylcholine. All of them increase the permeability of the receptor-rich microsacs to $^{22}Na^+$, i.e., behave as agonists. On the other hand, the exposure to antagonists, such as *d*-tubocurarine or gallamine triethiodide, which block the permeability response to agonists, is not followed by a significant change of their own affinity. The bisquaternary ligands decamethonium and hexamethonium fall into a particular category since they have little, if any, agonist effect *in vitro* (12) but nevertheless cause the change of affinity. In any case, the affinity change, once triggered, concerns primarily the interaction of the agonists with the receptor, whereas it has little, if any, effect on the binding of antagonists.

In the isolated membrane fragments, at rest, the receptor protein would therefore exist spontaneously in a low affinity state. The cholinergic agonists would then regulate their own affinity through the stabilization of a high affinity state.

As already mentioned: (*1*) the apparent dissociation constants measured *in vitro* by following $^{22}Na^+$ efflux coincide with the equilibrium binding constants of the low (rather than of the high) affinity state of the receptor protein (11, 12), and (*2*) the exposure of the membrane fragments to agonists is accompanied by a progressive decrease of the permeability response with a time course similar to that of the affinity change (min range), a phenomenon which closely resembles pharmacological desensitization (13, 15). Therefore, the low affinity state of the receptor protein, present in the membrane at rest, would be the functional one, i.e., a state susceptible to activation, whereas the high affinity state (17, 18) would correspond to a "desensitized" state of the receptor in which the ionophore is shut.

An interesting situation is offered by hexamethonium, an antagonist, which causes a much larger increase of affinity for an agonist phenylmethylammonium than for itself. A possible interpretation of this effect is that hexamethonium stabilizes the receptor protein in its desensitized state and, therefore, behaves like the "metaphilic" agents of Rang and Ritter (19).

A striking feature of the observed change of affinity is that it concerns a wide spectrum of agonists and seems independent of the precise structure of the compound which triggers it. It might even occur spontaneously in the absence of triggering ligand as if the transition was taking place between a small number of discrete states which preexist the binding of ligand. Similar slow transitions between discrete conformational states have been extensively reported with a number of cytoplasmic regulatory enzymes (20). With several of them, stabilization of the protein into one of these affinity states can be achieved, for instance, after chemical modification. In the present situation, this might happen during storage of the membrane fragments. The fact that local anesthetics (9) or lipid analogs (21) stabilize the high affinity state of the membrane-bound receptor suggests that the close lipid environment of the receptor protein might control this regulatory transition as well.

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