Conformational properties of the acetylcholine receptor as revealed by studies with constrained depolarizing ligands

(activators/inhibitors/dimensions of binding sites/binding site template/ligand flexibility)

N. H. WASSERMANN*, E. BARTELS†, AND B. F. ERLANGER*‡

*Department of Microbiology, Columbia University, Cancer Center/Institute of Cancer Research, New York, New York 10032; and †Universidad del Valle, Division de Salud, Departamento de las Ciencias Fisiologicas, Cali, Colombia, S.A.

Communicated by David Nachmansohn, November 6, 1978

ABSTRACT Conformational aspects of the acetylcholine receptor (AChR) of *Electrophorus electricus* have been examined by studies of its interaction with structurally related, constrained aromatic bis quaternary compounds. Among the compounds synthesized was 3,3'-bis[α -(trimethylammonium)methyl]azobenzene dibromide (3,3'-bisQ). This compound is photochromic and can exist in a *cis* or *trans* isomeric form, both of which have now been isolated in pure form. *Trans*-3,3'-bisQ is the most potent activator known, producing a 60-mV depolarization at 0.2 μ M and 50% activity at 0.06 μ M. The *cis* isomer is less than 1% as active. Its high activity and constrained structure suggest that *trans*-3,3'-bisQ can be considered to be a "template" of the combining site of AChR, when the latter is in the activated state. The following conclusions can then be drawn concerning the AChR binding site. (i) Depolarization can occur by interaction with reagents that are essentially inflexible. (ii) The binding site has a planar hydrophobic region that interacts with methylene groups of acetylcholine and with hydrophobic areas in general. (iii) In the same plane as the hydrophobic area is a site that interacts with electron-donating functional groups including the carbonyl oxygen of acetylcholine and the azo nitrogens of *trans*-3,3'-bisQ. (iv) About 1.5 Å out of the plane of the hydrophobic and the electron acceptor site is an anionic site; when the AChR is in the activated state, this site is separated from the electron acceptor site by 5.2 Å and from another anionic site by 11 Å. (v) The anionic sites are located within a cleft of limited size, sufficient to accommodate quaternary methyl groups. (vi) Although depolarization can occur with reagents that possess only hydrophobic and cationic groups if their geometric arrangement is proper, the highest activity resides in compounds capable of all of the interactions cited above.

Numerous attempts have been made to correlate the three-dimensional structures of compounds that activate or block the acetylcholine receptor (AChR) in such systems as electroplax preparations of *Electrophorus electricus* and motor end plates of frog muscle (1-5). Implicit in all of these studies is the existence of a protein-containing receptor that, when bound to a suitable ligand, regulates the permeability of and, hence, the electrical potential across an electrogenic membrane (6). A number of laboratories have reported successful isolation of the AChR protein (7-12) and have examined its properties both when free and when incorporated into membranes.

As with studies on enzymes, it is assumed that knowledge of the three-dimensional structure of active ligands can lead to an understanding of the topography of the binding site(s) of AChR and, hence, to an advance in the understanding of the mechanism by which electrogenic membranes are regulated. The more potent the agent, the more relevant is a study of its structure to the biochemical mechanism of the biological process, especially if the molecule is highly constrained (13).

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We have reported (14) on a structurally constrained, photochromic compound, *trans*-3,3'-bis[α -(trimethylammonium)methyl]azobenzene dibromide (*trans*-3,3'-bisQ), which was found to be one of the most potent activators of the AChR of *E. electricus*. Its activity (as measured by the concentration required for a 50% response) was about 500-fold greater than that of carbamylcholine, 50-fold greater than that of acetylcholine, and about 20-fold greater than that of decamethonium (15, 16). The highly specific nature of its interaction with AChR was substantiated by the finding that partial conversion to the *cis* isomer, by exposure to light of 365 nm, resulted in a marked diminution of activity. At that time, we were unable to isolate and therefore to test the purified *cis* isomer. This has now been accomplished; *cis*-3,3'-bisQ, as predicted (14), showed no depolarizing activity up to a concentration of 1 μ M; at most it is 1% as active as the *trans* isomer. (H. A. Lester, personal communication). Moreover, a number of related compounds have been synthesized and tested. The results of the activity studies are reported in this paper, as are conclusions concerning the structure of AChR that stem from these studies. The synthetic procedures will be described elsewhere.

OBSERVATIONS

Shown in Table 1 are the compounds prepared and tested for activity in the electroplax system. They represent several classes of aromatic bis-quaternary derivatives that differ in the manner in which two aromatic moieties are bridged. The class with the most numerous compounds is bridged by an azo group. Represented in this class are a series of bis-quaternary methyl derivatives that are isomers of bisQ. There are also a series of isomeric azo compounds in which one of the methyls of each quaternary moiety is replaced by a benzyl group. In a third class are three isomers of "diketo bisQ" in which diketo bridges

$\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{—C—C—} \end{array}$ link the aromatic quaternary bases. Finally, we used an analog of the highly potent activator, *trans*-3,3'-bisQ amide, in which an amide bond replaces the azo bridge.

Of the compounds tested, *trans*-3,3'-bisQ was the most potent activator of AChR, producing a 60-mV depolarization at 0.1 μ M. Its 3,3'-*cis* isomer was biologically inactive at 1 μ M, showing no activation or inhibition of carbamylcholine. At 3.5 μ M, depolarization of <6 mV occurred. This could be accounted for by the presence of about 1% *trans* isomer in the *cis* preparation. The positional isomers 2,2'- and 4,4'-bisQ were inhibitors of the action of carbamyl choline on AChR (see ref. 14 for procedures); 4,4'-bisQ was also a partial activator, 4 μ M

Abbreviations: AChR, acetylcholine receptor; bisQ, 3,3'-bis[α -(trimethylammonium)methyl]azobenzene dibromide.

‡ To whom reprint requests should be addressed.

Table 1. Compounds synthesized and tested

Compound*	—A—B—	R	AcChoR Activity		Max depolar., mV
			App. K_i , $\mu\text{M}^\dagger$	App. K_a , $\mu\text{M}^\dagger$	
3,3'-bisQ	—N=N—	—CH ₂ —N(CH ₃) ₃	No activity	0.06	60
3,3'-bisQ(<i>cis</i>)	—N=N—	—CH ₂ —N(CH ₃) ₃	No activity	(at 1 μM) [‡]	—
2,2'-bisQ	—N=N—	—CH ₂ —N(CH ₃) ₃	0.5	No activity	—
4,4'-bisQ	—N=N—	—CH ₂ —N(CH ₃) ₃	2	4	10
2,2'-bisQ benzyl	—N=N—	—CH ₂ —N(CH ₃) ₂ (CH ₂ φ)	Inhibitor (10 μM)	—	—
3,3'-bisQ benzyl	—N=N—	—CH ₂ —N(CH ₃) ₂ (CH ₂ φ)	Inhibitor (10 μM)	—	—
4,4'-bisQ benzyl	—N=N—	—CH ₂ —N(CH ₃) ₂ (CH ₂ φ)	Inhibitor (10 μM)	—	—
2,2'-Diketo bisQ		—CH ₂ —N(CH ₃) ₃	10	No activity	—
3,3'-Diketo bisQ		—CH ₂ —N(CH ₃) ₃	2.5	5	6
4,4'-Diketo bisQ		—CH ₂ —N(CH ₃) ₃	No activity	4	40
3,3'-bisQ amide		—CH ₂ —N(CH ₃) ₃	No activity	20	45

* Where applicable, all compounds are in the *trans* configuration unless designated otherwise.

[†] App. K_i is inhibition constant; app. K_a is activation constant; both are described in ref. 17.

[‡] Assay carried out by H. A. Lester, California Institute of Technology.

causing a depolarization of 10 mV. Substitution of benzyl groups for methyls on the quaternary nitrogens eliminated activating activity of the *trans*-3,3' derivative, converting it to an inhibitor. The analogous 2,2' and 4,4' derivatives were also inhibitors.

Of considerable interest is the respectable activity of 3,3'-bis[α -(trimethylammoniummethyl)]benzanilide dibromide (3,3'-bisQ amide), in which a peptide bond replaces the azo bridge and which, because of the *trans* double bond character of the amide bond, can assume the same conformation as *trans*-3,3'-bisQ. Although less active than the latter, it was as active as carbamyl choline (15, 16).

Also active as activators were the 3,3' and 4,4' isomers of diketo-bisQ. The 3,3' isomer depolarized only slightly (6 mV); the 4,4' isomer caused a potential change of 40 mV at 10 μM . The 3,3' isomer was an inhibitor of carbamylcholine.

Although not described here, another activator of the electrophax was 3-(α -bromomethyl)-3'-[α -(trimethylammonium)-methyl]azobenzene bromide (14). At a concentration of 0.5 μM , it produced a change in potential of 3 mV. When allowed to combine covalently with a sulfhydryl group in AcChoR (via displacement of Br), it caused a decrease in potential of 15 mV; this decrease could be made smaller by exposure of the system to light of 320 nm, presumably owing to conversion to the *cis* isomer.

COMMENT

We can conclude that the geometric arrangement of the quaternary groups—i.e., their size and position in space relative to the bridge joining the two aromatic groups—is one determinant for depolarizing activity. Our results are also consistent with the presence in the active compounds of at least one electron donor as part of the bridge structure, as demonstrated by the activity of bisQ amide and the 3,3' and 4,4' isomers of the diketo derivatives. On the other hand, the 2,2' diketo derivative was an inhibitor. Thus, the ability to donate electrons (or form hydrogen bonds) appears to play a part but only if the geometry is correct.

The most active depolarizer was *trans*-3,3'-bisQ. Its three-

dimensional structure, for the most part, is predetermined. The two benzene rings and the azo groups are in the same plane. From space-filling models, it is apparent that the only degrees of freedom that exist occur by rotation of the quaternary methylene groups around the bonds joining them to the benzene rings. One unknown is the relative positions of the quaternary groups. They can be "cisoid" or "transoid" with respect to one another. Charge repulsion and the likelihood that the molecule is symmetrical suggest that they are on opposite sides of the molecule. In that case, the distances between the two positively charged quaternary groups can be made to vary from 11.7 to 11.2 Å. In doing so, their distances from the nearest nitrogen of the azo group (the one linked to the opposing phenyl group) vary from 5.4 to 5.1 Å. If the quaternary nitrogen is placed about 1.3 Å above the plane of the benzene rings and of the azo bridge (which contains the electron donors), the N⁺—N⁺ distance becomes 11.2 Å and the N⁺—N(azo) distance becomes 5.2 Å. This geometry is closely similar to that of acetylcholine, in which the distance between the quaternary nitrogen and the carbonyl oxygen (the electron donor) is 5.15 Å and the positively charged quaternary nitrogen is 1.3 Å above the plane of the rest of the molecule (1). This geometry can be duplicated in 3,3'-bisQ benzyl but it has bulky benzyl groups and is a potent blocking agent rather than an activator.

Unlike *trans*-3,3'-bisQ, the 2,2' isomer is not an activator. Because of steric interference between the closely spaced quaternary groups, it cannot assume a planar conformation. On the other hand, the 4,4' isomer is planar and is a weak agonist. Its N⁺—N(azo) and N⁺—N⁺ distances (6.7 Å and 12.8 Å, respectively) are greater than they are in the highly active 3,3' isomer.

The weakly depolarizing compounds 3,3'-diketo bisQ and 4,4'-diketo bisQ cannot assume the geometry of the 3,3' and 4,4' azo compounds. Although the carbonyl groups are *trans* to each other, the molecules are not planar. They are "skew molecules" in which the two phenylketo halves are in planes that are at an angle to one another, of perhaps as much as 90°–100° (18, 19). This distortion from planarity may explain the decreased de-

FIG. 1. (A) Diagram of two acetylcholine (AcCho) molecules (open circles) and *trans*-3,3'-bisQ (solid circles) showing overlapping of critical functional groups. (B) Space-filling models of the same molecules. Two acetylcholine molecules are suspended above *trans*-3,3'-bisQ. One methyl group has been removed from each of the quaternary nitrogens.

polarization caused by these compounds. Each half of the 3,3' isomer bears a close resemblance to acetylcholine with respect to the arrangement in space of the carbonyl oxygen and the quaternary nitrogen. In fact, they are separated by 5.2 Å, exactly as they are in acetylcholine and, incidentally, as they are in another activator, prostigmine. The reason for the higher activity of the 4,4' isomer is not clear. The N⁺-O(carbonyl) distance is about 7.5 Å, considerably greater than in acetylcholine. However, if bis-quaternary derivatives can depolarize the electrogenic membrane by spanning sites on neighboring receptor molecules, then additional information will have to be obtained relative to the geometric arrangement of the receptors in the membrane.

Distortion from planarity is more marked in *cis*-3,3'-bisQ which, unlike the *trans* isomer, is inactive. The molecule is neither planar nor symmetrical (20, 21). The two benzene rings are in planes at an angle of 53° from one another.

Our findings are not consistent with the suggestion of Pauling and Petcher (3) that flexibility is a required property of a depolarizing compound and that inhibitors are constrained. The active compounds described in this paper are highly constrained. This is particularly true for *trans*-3,3'-bisQ and, indeed, it is this constraint that is likely to be responsible for its high activity—i.e., its geometry is closely similar to that of the binding site of AcChoR in its active, depolarized state which, presumably, it stabilizes. Our findings also do not agree with the suggestion of Pauling and Petcher that in the "resting state" the sites on the quaternary groups are about 11 Å apart and that they move closer together upon depolarization. The high activity of *trans*-3,3'-bisQ would suggest that the N⁺-N⁺ distance is about 11 Å in the activated state. The dimensions in the inactive, resting state cannot be deduced from our data. With respect to acetylcholine, the implication would be that flexibility of structure plays no role in its mechanism of action. In their paper, Pauling and Petcher (3) attempted to explain why certain compounds were activators and others were inhibitors. Their treatment of this problem assumed that both types of reagents interacted with the same binding site on the receptor. The work of Fu *et al.* (22), however, provides evidence that depolarizers and blocking agents bind to different sites. Our studies, therefore, bear on the topography of the binding site for activators.

Fig. 1 shows the conformations of two acetylcholine molecules and *trans*-3,3'-bisQ placed so that the functional groups

that interact with the same sites of AcChoR are in line with one another. The quaternary nitrogen groups of the two compounds are superimposable as are the azo nitrogens with the carbonyl oxygens of the two acetylcholine molecules. Also shown is overlap between a portion of the aromatic rings of the azo compound and methylene carbons of acetylcholine. From this we can conclude the following about the binding site of the AcChoR when it is in the activated state.

i. It has a planar hydrophobic region (23) that interacts with a methylene group of acetylcholine or with hydrophobic areas in general.

ii. In the same plane as the hydrophobic area is a site that can interact with functional groups that can donate electrons—the azo groups in *trans*-3,3'-bisQ or the carbonyl of the ester functional group in acetylcholine. With respect to the azo nitrogens of *trans*-3,3'-bisQ, preliminary resonance Raman spectroscopy supports the suggestion of an interaction of these groups with membrane-bound AcChoR (A. Lewis, personal communication).

iii. About 1.5 Å out of the plane of the hydrophobic and the electron acceptor site is an anionic site located in a cleft of limited size, sufficient to accommodate quaternary methyl groups but not quaternary benzyl dimethyl groups. This anionic site is about 5.2 Å from the electron acceptor site. According to Pullman *et al.* (24), the nitrogen of the quaternary group of acetylcholine is nearly neutral. Seventy percent of the positive charge is distributed among the methyl groups, forming a spherical field of positive electricity. Thus, the location of the anionic site below the plane of the other binding loci allows for accommodation of this positively charged sphere. This arrangement can explain why unsubstituted amines do not depolarize—e.g., bromoacetylcholine is an affinity alkylating agent of AcChoR but not an activator (25).

Highest activity occurs when an activator has the functional groups and geometry for all three interactions to occur. Although the activity of decamethonium suggests that interaction with the hydrophobic and with the cationic sites is sufficient for depolarization, additional energy of binding is contributed by the electron donors. Thus, *trans*-3,3'-bisQ, with two electron donors, is active at lower concentrations than 3,3'-bisQ amide which has only one. Both depolarize to a similar extent at saturation. This description agrees with and extends the suggestion of others (e.g., refs. 5 and 6). Its uniqueness lies in the fact that the geometry of the binding site of AcChoR was derived from

studies on highly potent activators (in one case, the most potent one known) with highly constrained structures, which, in the case of *trans*-3,3'-bisQ, can be considered as a template for the AcChoR combining site(s) in the active, depolarizing state.

These studies were funded by National Science Foundation Grant PCM-77-02140.

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