Proc. Nat. Acad. Sci. USA
Vol. 68, No. 8, pp. 1820–1823, August 1971

Photochromic Activators of the Acetylcholine Receptor

(*Electrophorus electricus* electroplax/membrane potential/photoregulation/vision)

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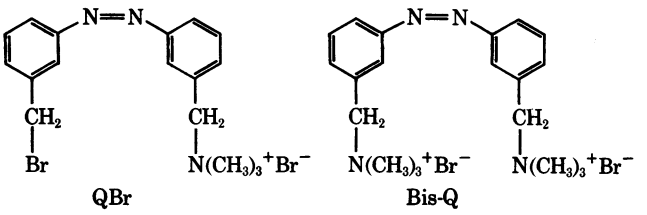
Communicated by David Nachmansohn, May 19, 1971

ABSTRACT **Two photochromic activators of the electrogenic membrane of the electroplax of *Electrophorus electricus* are described. *Trans*-3,3′-bis[α-(trimethylammonium)methyl]azobenzene dibromide (Bis-Q), one of the most potent ever reported, is active at concentrations of less than 10^{-7} M. Its *cis* isomer, which is obtained from the *trans* by exposure to light of 330 nm, is practically devoid of activity. Photoregulation of the potential of the membrane takes place in the presence of Bis-Q, presumably because of the conversion of the active *trans* isomer to the inactive *cis* isomer in the single-cell electroplax system. The second activator, 3-(α-bromomethyl)-3′-[α-(trimethylammonium)methyl]azobenzene bromide (QBr) can be covalently attached to the electroplax membrane after reduction of the membrane with dithiothreitol. Activation of the membrane is induced by the covalently linked reagent. Its *cis* isomer, obtained from the *trans* by exposure to light of 330 nm, is, like *cis*-Bis-Q, of very low activity. Both isomers of Bis-Q are equally active as inhibitors of acetylcholinesterase, 50% inhibition occurring at a concentration of 10^{-5} M. The possibility of using *trans*-Bis-Q and *trans*-QBr to characterize and isolate the receptor protein is discussed.**

Systems in which photoregulation could be studied at the molecular level were described in previous papers. In these systems, photochromic azo derivatives were used as effector molecules to regulate the activities of chymotrypsin (1) and acetylcholinesterase (2, 3) and to photoregulate the potential of the excitable membrane of the monocellular electroplax preparation (4). Photoregulation was achieved by exploiting differences between the biochemical activities of the *cis* and *trans* isomers of the photochromic compounds, the relative concentrations of which were influenced by the wavelength of light to which the solution was exposed [or light vs. darkness, in one case (3)].

Light-induced changes in potential of the electroplax membrane may be considered as a model for the process of vision, in which the *cis* to *trans* isomerization of retinal is the first step in the initiation of a neural impulse. In the latter case, however, as well as in the phytochrome system of plants (5), the photochromic substances are located intracellularly, making for a highly efficient process. It thus appeared of interest to prepare a light-sensitive ligand that would form a covalent bond with the receptor protein of the electroplax. A compound with the desired properties was prepared: 3-(α-bromomethyl)-3′-[α-(trimethylammonium)methyl]azobenzene (QBr). Also synthesized was the closely related 3,3′-bis[α-(trimethylammonium)methyl]azobenzene (Bis-Q), the *trans* isomer of which was found to be a potent receptor activator, one of the most potent thus far described.



The high affinity and specificity of Bis-Q may make it a useful reagent for the characterization, isolation, and purification of the receptor protein. Some experiments with the two azo compounds are presented in this paper.

METHODS

Preparation of 3,3′-bis(α-bromomethyl)azobenzene

2 g (9.5 mmol) of 3,3′-dimethylazobenzene (K & K Laboratories), 5 g (28.1 mmol) of *N*-bromosuccinimide (Fisher Scientific) and 60 mg of benzoyl peroxide in 40 ml of dry carbon tetrachloride were refluxed vigorously for 2 hr, the reaction mixture being protected from external moisture. Another 60-mg portion of benzoyl peroxide was then added and refluxing was continued for two more hours. The reaction mixture was allowed to stand overnight at room temperature, after which the insoluble succinimide was removed by filtration and washed with three 15-ml portions of dry carbon tetrachloride. The combined filtrates were distilled to dryness under reduced pressure and the orange crystalline residue (3.6 g) was stirred with 50 ml of anhydrous methanol. The crystals were recovered and dried in a desiccator. Yield 1.56 g, mp 137–139°C. This product is pure enough to be used for the next step.

Recrystallization twice from methanol yielded a product with mp 143°C.

Calcd for $C_{14}H_{12}N_2Br_2$ (368.1): C, 45.68; H, 3.29; N, 7.61; Br, 43.42. Found: C, 45.45; H, 3.47; N, 7.39; Br, 43.03.

Preparation of 3,3′-bis[α-(trimethylammonium)methyl]-azobenzene bromide (Bis-Q)

3,3′-Bis(α-bromomethyl)azobenzene (1.1 g, 3 mmol) was covered with 20 ml of 25% trimethylamine in methanol (85 mmol) in a 100-ml flask with a stirring bar. The flask was stoppered with a polyethylene (or Teflon) stopper and the mixture was stirred for 2 hr at room temperature. An additional 20 ml of methanol was added, and the stirring was re-

Abbreviations: Bis-Q, *trans*-3,3′-bis[α-(trimethylammonium)-methyl]azobenzene dibromide; QBr, 3-(α-bromomethyl)-3′-[α-(trimethylammonium)methyl]azobenzene bromide.

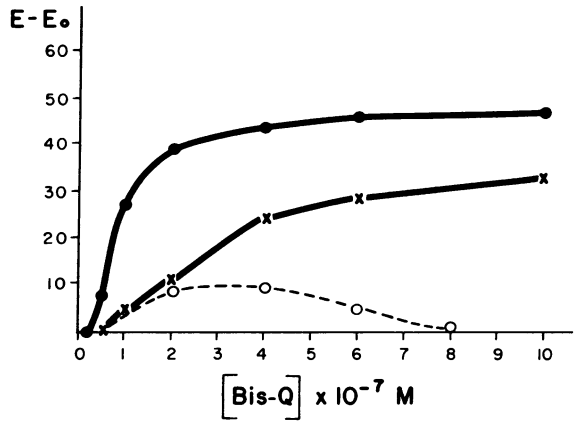


FIG. 1. Dose–response curves of *trans*-Bis-Q (●), "*cis*-equilibrium mixture" (×), and *cis*-Bis-Q (calculated) (○).

sumed until the orange leaflets dissolved completely. The solution was kept at room temperature overnight without stirring. Excess amine and solvent were removed by distillation *in vacuo*. The crystalline orange residue was dissolved in 15 ml of methanol and poured slowly into 300 ml of ethyl ether, with stirring. The quaternary salt precipitated and was recovered by filtration. Recrystallization was from warm methanol–ether 2:1. The product crystallized as orange leaflets. Yield, 0.95 g; mp 252–253°C.

Calcd for $C_{20}H_{30}N_4Br_2$ (486.3): C, 49.39; H, 6.22; N, 11.54; Br, 32.86. Found: C, 49.43; H, 6.24; N, 11.39; Br (total) 32.85; Br^-, 32.70.

The ultraviolet spectrum of a 5% methanol–95% water solution had a maximum at 318 nm, ϵ_{max} 20,700. Its appearance was typical of a *trans*-azobenzene isomer. Exposure to ultraviolet light with a maximum at 360 nm (Spectroline B-100) caused conversion to the *cis* isomer. However, it was never possible to effect more than about 85% conversion. Attempts to isolate the pure *cis* isomer have, as yet, not been successful. Therefore, it must be kept in mind that solutions used in experiments carried out with *cis* isomer had about 15% *trans* isomer present. This equilibrium mixture was stable in the dark, but exposure to visible light (Photoflood) caused conversion to an equilibrium mixture of about 90% *trans*–10% *cis* isomer.

3-(α-Bromomethyl)-3′-[α-(trimethylammonium)methyl]-azobenzene bromide (QBr)

3,3′-Bis(α-bromomethyl)azobenzene (1.47 g, 5 mmol) was dissolved in 50 ml of boiling methanol in a 100-ml flask. After cooling to about 35°C, 0.12 g (2 mmol) of trimethylamine (0.5 ml of a 25%, w/v, methanolic solution) was added in two portions at 15-min intervals, with stirring. The stoppered flask was kept at room temperature overnight.

Without removing some precipitated crystals, we reduced the volume under reduced pressure to about 15 ml, and placed the flask in a freezer for 3 hr. Most of the excess 3,3′-bis(α-bromomethyl)azobenzene precipitated and was removed by filtration. The filtrate was poured slowly, with stirring, into 300 ml of dry ethyl ether in order to precipitate the quaternary salt. After 2 hr in the freezer, the crystals were recovered by filtration, washed with ethyl ether, and dried at 37°C. Yield, 665 mg, mp 177–8°C. Recrystallization from methanol–ether

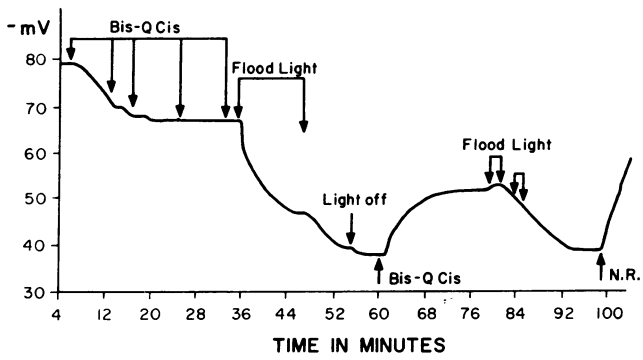


FIG. 2. Photoregulation of electrogenic membrane in presence of Bis-Q, 1×10^{-7} M. "Bis-Q *cis*" refers to the "*cis*-equilibrium mixture" described in text. N.R., normal Ringer's solution.

1:2 (with cooling in a Dry Ice bath) raised the melting point to 192–3°C (decomp.) Yield, 0.48 g.

Calcd for $C_{17}H_{21}N_3Br_2$ (427.21): C, 47.79; H, 4.96; N, 9.83; Br, 37.41; Br^-, 18.70. Found: C, 47.34; H, 5.28; N, 9.71; Br, 37.04; Br^-, 19.06.

The ultraviolet spectrum in 5% methanol had a maximum at 319 nm, ϵ_{max} 20,000. Exposure to ultraviolet light with a maximum at 360 nm (Spectroline B-100) caused conversion to an equilibrium mixture of about 90% *cis*–10% *trans* isomers. This mixture was stable in the dark but was converted by the light of a Photoflood to 15% *cis*–85% *trans*.

Electroplax experiments

Details of the methods used are given in previous papers (4, 6, 7).

RESULTS

Fig. 1 shows the dose–response curves of *trans*-Bis-Q and of the equilibrium mixture obtained by ultraviolet irradiation (referred to as "*cis*-equilibrium mixture"). Also shown is the calculated curve for the pure *cis* isomer, assuming the presence of 15% *trans* isomer in the equilibrium mixture (see *Methods*). The concentration at half-maximal response ($6–8 \times 10^{-8}$ M) was used for comparison, the *trans* isomer was found to be 500 times more potent than carbamylcholine (8). The maximal response is smaller, being comparable to that of decamethonium (8). The rates of depolarization and of recovery are slower than in the presence of carbamylcholine. At concentrations of 2×10^{-6} M or higher, repolarization of the membrane occurs. The calculated activity for the *cis* isomer indicates very low potency; it is possible that pure *cis* isomer, when isolated, may even lack activity.

Fig. 2 shows the effect on the cell of the "*cis*-equilibrium mixture" at a concentration of 10^{-7} M. After a steady state was reached, the preparation was exposed to a Photoflood lamp. A further depolarization occurred, which could be reversed by applying the "*cis*-equilibrium mixture" again. A second exposure to the Photoflood caused a second decrease in membrane potential.

The activity of *trans*-Bis-Q is inhibited by curare (Fig. 3). Calculation of the dissociation constant for curare yields a value of 1.5×10^{-7}, in agreement with the value obtained from studies with carbamylcholine (9). Increase of the concentration of *trans*-Bis-Q to 2×10^{-6} M in the presence of

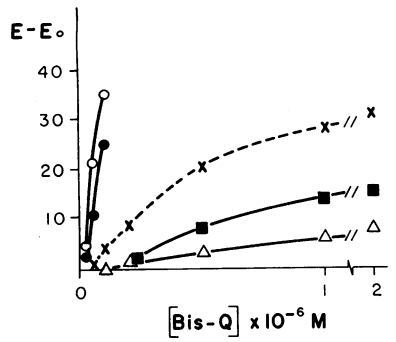


FIG. 3. Action of *trans*-Bis-Q in presence of curare. *Trans*-Bis-Q alone (○) and (●) (two different cell preparations); *trans*-Bis-Q and 5×10^{-7} M curare, (×); *trans*-Bis-Q and 10^{-6} M curare, (■); *trans*-Bis-Q and 5×10^{-6} M curare, (△).

curare causes repolarization, as it did in its absence, which indicates the possible existence of two binding sites, only one of which leads to competition with the binding of curare. At low concentrations of *trans*-Bis-Q, its activity and that of carbamylcholine are additive; at high concentrations of the former ($>2 \times 10^{-6}$ M), repolarization always occurs even in the presence of carbamylcholine. Reduction of the receptor sites with dithiothreitol inhibits the response to *trans*-Bis-Q.

Exposure of a cell to *trans*-QBr at a concentration of 2×10^{-7} M causes a depolarization of about 5–10 mV. The action potential is reduced, but it and the resting potential recover when the cell is washed with Ringer's solution. Increase of the concentration to 10^{-5} M causes repolarization of the membrane, but the action potential is blocked. *trans*-QBr inhibits the cell's response to carbamylcholine. One can calculate a dissociation constant for *trans*-QBr of 4×10^{-7}, but this can only be considered an approximation since the maximal depolarization is reduced considerably and is reached at a low concentration of carbamylcholine. The inhibition is completely reversible upon removal of the compound.

Prior treatment with dithiothreitol results in irreversible inhibition of the effect of carbamylcholine by *trans*-QBr, as shown in Fig. 4. First depolarization by carbamylcholine is

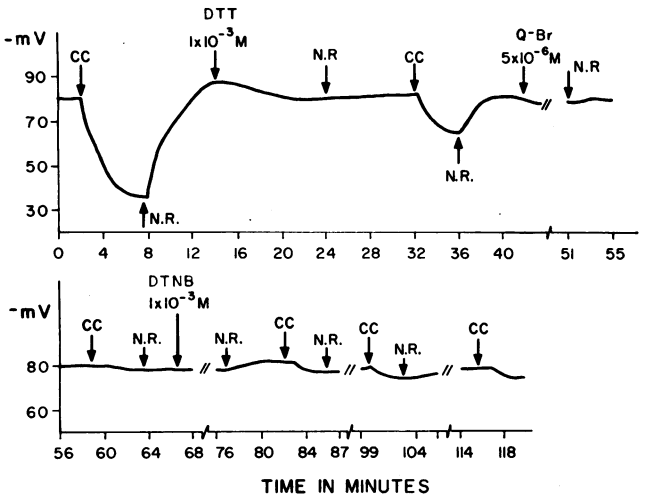


FIG. 4. Covalent linkage of QBr to reduced membrane. CC, 5×10^{-6} M carbamylcholine; N.R., normal Ringer's solution; DTT, dithiothreitol; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid). See text for description of experiment.

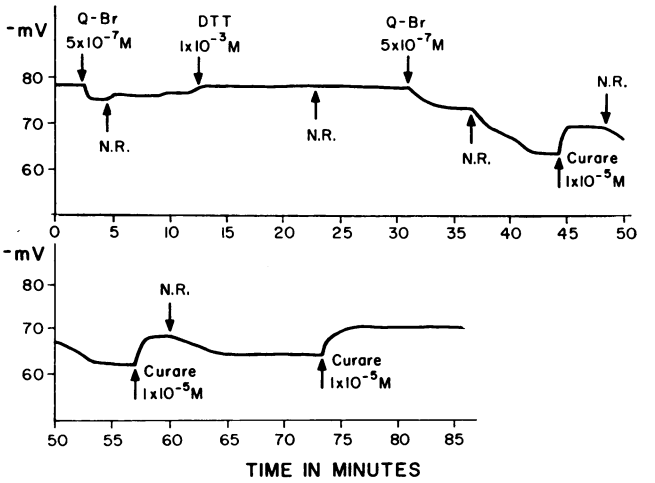


FIG. 5. Further experiments showing effects of linking QBr covalently to the reduced membrane (see legend of Fig. 4 and text for abbreviations and for description of the experiment).

shown. After treatment of the cell with dithiothreitol, carbamylcholine still causes depolarization, but to about one-third the extent. If the reduced cell is now exposed for 10 min to 5×10^{-6} M *trans*-QBr (followed by Ringer's solution), the response to carbamylcholine is abolished. Treatment with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), which normally reoxidizes the reduced receptor and restores the cell's sensitivity to carbamylcholine, is without effect after exposure to *trans*-QBr. If the dithiothreitol-reduced cell was exposed to a mixture of the *trans*-QBr and curare (10^{-5} M), subsequent treatment with DTNB causes a partial recovery of the response to carbamylcholine. These results indicate that *trans*-QBr can form a covalent bond with a sulfhydryl group of the reduced receptor, comparable to that formed by bromoacetylcholine, as reported by Silman (10) and by Silman and Karlin (11).

The action of *trans*-QBr is elaborated further in Fig. 5. Before dithiothreitol treatment, *trans*-QBr (5×10^{-7} M) depolarizes the membrane by 3 mV; this can be reversed with Ringer's solution. After treatment of the cell for 10 min with dithiothreitol at 10^{-3} M, a 5-mV depolarization occurs with 5×10^{-7} M of *trans*-QBr. Washing with Ringer's solution, to remove excess unreacted compound, causes an additional depolarization and a blocking of the action potential, as well. Exposure to curare (10^{-5} M) or reapplication of *trans*-QBr at a higher concentration repolarizes the cell and restores the action potential.

Preliminary experiments indicate that *cis*-QBr is less active than the *trans* isomer.

DISCUSSION

The acetylcholine receptor protein present in the excitable membrane, by its reaction with acetylcholine, controls permeability changes that allow ion movements during the generation of the bioelectric impulse. A small number of related compounds, e.g., carbamylcholine, decamethonium, and phenyltrimethylammonium may perform the same function. Other compounds bearing quaternary methyl groups also interact with the receptor but act as antagonists, e.g., curare. An analogy can be drawn between the interaction of ligands with the receptor and similar interactions in the field of

enzymology. There are many more inhibitors for a particular enzyme than there are substrates, since the structural requirements of the former (such as conformation, electron distribution, functional groups, etc.) are less stringent. Consequently, less information about the topography and properties of the enzyme can be obtained from a study of enzyme-inhibitor reactions than can be obtained by an examination of enzyme–substrate interactions. Similarly, there are many more receptor inhibitors than there are activators, and the latter can yield more information about the characteristics of the receptor protein.

The finding that *trans*-Bis-Q at exceedingly low concentrations (about 10^{-7} M) depolarizes the electrogenic membrane of the electroplax indicates that it is highly specific for the receptor protein. The exquisite requirements of this specificity are emphasized by the finding that the *cis* isomer has very little (or no) activity. Moreover, in a single electroplax membrane there is, according to the figures of Changeaux *et al.* (12), about 1×10^{-13} mol of acetylcholinesterase (according to more recent and more elaborate evaluations of Dr. T. Rosenberry, personal communication, the figure is 5×10^{-13} mol). Let us assume that the number of receptor molecules is the same as previous estimates indicate (13); let us further assume that the number of receptor molecules located in synaptic junctions is about 5–10% of the total surface area of the excitable membrane. *Trans*-Bis-Q would reach the receptor in the membrane only at the level of the junctions (14, 15). Since there are about 20,000–40,000 junctions per electroplax cell, there would be about 5×10^{8} to 1×10^{9} receptor molecules at the junctional membranes. At 50% activation, a concentration of Bis-Q of 8×10^{-8} M or 8×10^{-11} mol/ml is required. This is equivalent to about 10^{13} molecules of *trans*-Bis-Q/ml of solution, the approximate volume of the solution in which the electroplax is bathed. Thus, about 10^{4} molecules of Bis-Q are required in the solution for the reaction with the receptor. *Trans*-Bis-Q, therefore, is highly specific for the receptor and might be useful in binding studies to estimate the number of receptor molecules in an electroplax preparation. Moreover, appropriate derivatives may aid in the isolation of the receptor by affinity chromatography.

The marked difference in activity between the *trans* and *cis* isomers made it possible to photoregulate the potential difference across the innervated membrane of the electroplax. A similar achievement was previously reported (4), in which a photochromic antagonist of acetylcholine was used. In the present case, photoregulation was by direct influence on the membrane receptor and is, therefore, likely to be more truly analogous to the process of vision, in which the membrane potential is directly affected by a *cis–trans* conversion of the retinal moiety of rhodopsin.

Preliminary measurements of the effect of Bis-Q on acetylcholinesterase show that 50% inhibition occurs at about 10^{-5} M. Moreover, there is no significant difference between the *cis* and *trans* isomers, which emphasizes the highly specific nature of the interaction between *trans*-Bis-Q and the receptor and provides good additional evidence that acetylcholinesterase and the receptor are distinct entities.

The covalent attachment of QBr to the membrane receptor was accomplished by the procedure reported by Silman (10) and Silman and Karlin (11) for receptor specific molecules. The ability of QBr to depolarize the membrane while covalently linked is similar to the effects reported by Silman and Karlin for covalent attached *p*-(carboxyphenyl)trimethylammonium iodide. Lower concentrations of QBr were used in our experiments: 5×10^{-7} M QBr compared with 10^{-4} M *p*-nitrophenylester of *p*-(carboxyphenyl)trimethylammonium iodide in the experiments of Silman and Karlin.

Preliminary experiments indicate that *trans*-QBr is more active than *cis*. Thus, we have the potential of photoregulating the electroplax membrane by means of a covalently linked effector molecule.

We thank Prof. D. Nachmansohn for his encouragement and many helpful discussions. This investigation was supported in part by the National Science Foundation grants NSF-GB-17915, NSF-GB-25362, NS03304, The New York Heart Association, The Hoffman-La Roche Foundation, and The Irene Heinz Given and John LaPorte Given Foundation, Inc.

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