Photochromic Activators of the Acetylcholine Receptor

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

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ABSTRACT Two photochromic activators of the electrionic 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 10⁻⁷ 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 dibromide; QBr, 3-(α-bromomethyl)-3'-[α-(trimethylammonium)methyl]azobenzene bromide.

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

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.

Caled for C₂₅H₁₇N₂Br₂ (368.1): C, 45.68; H, 3.29; N, 6.11; Br, 43.42. Found: C, 45.45; H, 3.47; N, 7.39; Br, 43.05.

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-
The ultraviolet spectrum of a 5% methanol–water solution had a maximum at 318 nm, \( \varepsilon_{\text{max}} \) 20,700. Its appearance was typical of a trans-azobenzene isomer. Exposure to ultraviolet light with a maximum at 360 nm (Spectrolite 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-(\( \alpha \)-Bromomethyl)-3'-\( \alpha \)-(trimethylammonium)methyl-azobenzene bromide (QBr)

3,3'-Bis(\( \alpha \)-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 stopped 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(\( \alpha \)-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

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\text{cis} \times 10^{-7} M
\]

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

The concentration of the cis isomer was estimated by ultraviolet and ultraviolet-visible absorption spectrophotometry. The cis isomer concentration was calculated as the percentage of the cis isomer in the mixture (see Methods).

The results showed that the cis isomer concentration was about 90% in the mixture. The cis isomer concentration was determined by the method described in the previous paper (4).

Electropax 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 (\( B=8 \times 10^{-4} \) 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 \( \times 10^{-4} \) 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 \( 1.5 \times 10^{-7} \), in agreement with the value obtained from studies with carbamylcholine (9). Increase of the concentration of trans-Bis-Q to \( 2 \times 10^{-4} \) M in the presence of
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 x 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 x 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 x 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 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 x 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^{-3} 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 sulphydryl 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 x 10^{-7} M) depolarizes the membrane by 3 mV; this can be reversed with Ringer's solution. After removal of trans-QBr from the cell for 10 min with dithiothreitol at 10^{-3} M, a 5-mV depolarization occurs with 5 x 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^{-6} 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 \textit{trans}-Bis-Q at exceedingly low concentrations (about $10^{-4} \text{M}$) depolarizes the electrogenic membrane of the electroplax indicates that it is highly specific for the receptor protein. The requisite requirements of this specificity are emphasized by the finding that the \textit{cis} isomer has very little (or no) activity. Moreover, in a single electroplax membrane there is, according to the figures of Changeux \textit{et al.} (12), about $1 \times 10^{-13}$ mol of acetylcholinesterase (according to more recent and more elaborate evaluations of Dr. T. Rosenberry, personal communication, the figure is $5 \times 10^{-11}$ mol). Let us assume that the number of receptor molecules in 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. \textit{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 $5 \times 10^4$ to $1 \times 10^5$ receptor molecules at the junctional membranes. At 50% activation, a concentration of Bis-Q of $8 \times 10^{-4} \text{M}$ or $8 \times 10^{-11} \text{mol/ml}$ is required. This is equivalent to about $10^{13}$ molecules of \textit{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. \textit{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 \textit{trans} and \textit{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 \textit{cis}–\textit{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 $10^{-3}$ M. Moreover, there is no significant difference between the \textit{cis} and \textit{trans} isomers, which emphasizes the highly specific nature of the interaction between \textit{trans}-Bis-Q and the receptor and provides good additional evidence that acetylcholinesterase and the receptor are distinct entities.

The covalent attachment of QB 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 QB to depolarize the membrane while covalently linked is similar to the effects reported by Silman and Karlin for covalent attached \textit{p}-(carboxyphenyl)trimethylammonium iodide. Lower concentrations of QB were used in our experiments: $5 \times 10^{-7}$ M QB compared with $10^{-4}$ M \textit{p}-nitrophenylester of \textit{p}-(carboxyphenyl)trimethylammonium iodide in the experiments of Silman and Karlin.

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

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