A Covalently Bound Photoisomerizable Agonist

Comparison with Reversibly Bound Agonists at Electrophorus Electroplaques

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ABSTRACT After disulphide bonds are reduced with dithiothreitol, *trans*-3-(α-bromomethyl)-3′-[α-(trimethylammonium)methyl]azobenzene (*trans*-QBr) alkylates a sulfhydryl group on receptors. The membrane conductance induced by this "tethered agonist" shares many properties with that induced by reversible agonists. Equilibrium conductance increases as the membrane potential is made more negative; the voltage sensitivity resembles that seen with 50 μM carbachol. Voltage-jump relaxations follow an exponential time-course; the rate constants are about twice as large as those seen with 50 μM carbachol and have the same voltage and temperature sensitivity. With reversible agonists, the rate of channel opening increases with the frequency of agonist-receptor collisions: with tethered *trans*-QBr, this rate depends only on intramolecular events. In comparison to the conductance induced by reversible agonists, the QBr-induced conductance is at least 10-fold less sensitive to competitive blockade by tubocurarine and roughly as sensitive to "open-channel blockade" by QX-222. Light-flash experiments with tethered QBr resemble those with the reversible photoisomerizable agonist, 3,3′,*bis*-[α-(trimethylammonium)methyl]azobenzene (Bis-Q): the conductance is increased by *cis* → *trans* photoisomerizations and decreased by *trans* → *cis* photoisomerizations. As with Bis-Q, light-flash relaxations have the same rate constant as voltage-jump relaxations. Receptors with tethered *cis*-QBr have a channel duration severalfold briefer than with the tethered *trans* isomer. By comparing the agonist-induced conductance with the *cis*/*trans* ratio, we conclude that each channel's activation is determined by the configuration of a single tethered QBr molecule. The QBr-induced conductance shows slow decreases (time constant, several hundred milliseconds), which can be partially reversed by flashes. The similarities suggest that the same rate-limiting step governs the opening and closing of channels for both reversible and tethered agonists.

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Therefore, this step is probably not the initial encounter between agonist and receptor molecules.

INTRODUCTION

According to present concepts, the nicotinic acetylcholine receptor has two functionally distinct regions, a binding site(s) for agonists and a channel for ions. Recent experiments show that receptor channels have a unique "open" conductance and that channels undergo the transitions to and from the open state in a very short time compared with the duration of the open state (Neher et al., 1978). The frequencies, or rate constants, of the opening and closing events are measured in experiments that exploit spontaneous or forced perturbations from equilibrium activation. These experiments reveal that the opening frequency increases with the agonist concentration, that the closing frequency increases with membrane depolarization, and that both frequencies depend strongly on temperature and on the structure of the agonist molecule.

What is the nature of the molecular step that limits the rate of these transitions? A simple view is that the binding and dissociation of agonist molecules themselves limit the rate of channel kinetics (Kordas, 1972 *a*, *b*). Alternatively, the agonist-receptor complex could undergo rate-limiting conformational changes while the agonist is bound (Magleby and Stevens, 1972 *a*, *b*). One might distinguish between these two schemes by experiments in which the agonist molecules are covalently bound at the binding site, assuming that such a modification does not change the mechanism of channel activation. If binding is normally rate-limiting, this treatment would profoundly alter the kinetics and voltage sensitivity of the agonist-induced conductance. On the other hand, the conformational-change theory predicts that channels would still open and close with roughly the same kinetics, voltage sensitivity, and temperature sensitivity as for reversibly bound agonists.

Experiments with such a permanently bound agonist would also provide a test of present theories about drug blockade. Curare and other competitive antagonists are thought to bind at the agonist site; but local anesthetics and other "open-channel blockers" are thought to interact primarily with the open channel, terminating it prematurely. The covalently bound agonist would be expected to reduce the blocking potency of the former drugs, but not that of the latter.

Unfortunately, no presently known drugs bind covalently directly at the agonist-receptor binding site. However, several agonists can be covalently linked to the receptor at a point near the binding site (Karlin, 1969; Silman and Karlin, 1969; Bartels et al., 1971; Erlanger, 1976; Cox et al., 1979 *a*, *b*). We term such compounds "tethered" agonists. Bartels et al. (1971, 1976) studied the properties of one such compound, *trans*-3-(α-bromomethyl)-3′-[α-(trimethylammonium)methyl]azobenzene (*trans*-QBr). This drug is a reversible partial agonist on normal *Electrophorus* electroplaques, but the following results showed that it binds irreversibly near the agonist binding site after receptors have been reduced by dithiothreitol (DTT). (*a*) Exposure to DTT followed by QBr causes a depolarization that cannot be reversed by extensive

washing. (*b*) Cells no longer respond to carbachol with further depolarization. (*c*) Tubocurarine (dTC) partially repolarizes the cell but the depolarization is reestablished when dTC is washed out. (*d*) Receptors can be partially protected against the irreversible activation if tubocurarine is present during the exposure to *trans*-QBr. (*e*) After reduction by DTT alone, receptors show reduced responses to many agonists. This blockade can be reversed by exposure to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). If, however, the cell is exposed to *trans*-QBr after reduction by DTT, subsequent DTNB treatment repolarizes the cell but fails to restore normal sensitivity (Bartels-Bernal et al., 1976). (*f*) Subsequent exposure to DTT now reestablishes the depolarization.

QBr presumably alkylates the same sulfhydryl group, formed from a disulfide bond by the dithiothreitol treatment, that reacts with several other compounds, including (*a*) the tethered agonists, bromoacetylcholine and the *p*-nitrophenyl ester of (*p*-carboxyphenyl)trimethylammonium (NPTMB) (Silman and Karlin, 1969; Chang et al., 1977); (*b*) the tethered blocking drugs, 4-(*N*-maleimido)phenyltrimethylammonium (MPTA; Karlin and Winnik, 1968) and 4-(*N*-maleimido)benzyltrimethylammonium (MBTA; Karlin and Cowburn, 1973); and (*c*) sulfite (Steinacker, 1979). The sulfhydryl group is on a receptor subunit of molecular weight 40,000 (Karlin and Cowburn, 1973; Sobel et al., 1977; Chang et al., 1977); there are two such subunits for each receptor monomer of molecular weight 250,000–290,000 (Raftery et al., 1976; Sobel et al., 1977; Reynolds and Karlin, 1978). In fish receptor, only one sulfhydryl group can be alkylated per monomer, although two molecules of elapid α-toxin bind specifically per monomer (Damle et al., 1978; Moore and Raftery, 1979).

QBr has the additional property that it can be photoisomerized between inactive and active configurations while bound to receptors; thus, the agonist-induced conductance can be regulated with light flashes. We have exploited several advantages of this technique. (*a*) Desensitization can be minimized by keeping the tethered QBr in the inactive state most of the time. (*b*) We have investigated the stoichiometry of receptor activation, and the possible functional role of interactions between receptor monomers, by measuring the conductance induced by known *cis/trans* ratios of tethered QBr molecules. (*c*) We have compared light-flash relaxations with tethered QBr and with the structurally related reversible photoisomerizable agonist, 3,3′, *bis*-[α-(trimethylammonium)methyl]azobenzene (Bis-Q; Bartels et al., 1971; Erlanger, 1976; Lester and Chang, 1977; Nass et al., 1978; Wassermann et al., 1979). Two abstracts have appeared describing the work (Lester et al., 1979 *a*, *b*).

METHODS

Electrical Arrangements

The voltage-clamp circuit measures (*a*) steady-state currents induced by cholinergic agonists and (*b*) conductance relaxations resulting from jumps of membrane voltage (Sheridan and Lester, 1977; Lester, 1978). Leakage and capacitative currents are determined by measurements in the absence of agonist or—even when agonist is present at the receptors—by measurements in a region of membrane potential where

there is little or no agonist-induced conductance (Lester et al., 1975). To reduce digitizing noise, the former 10-bit analog-to-digital converters have been replaced by 12-bit units.

Optical Arrangements

The optical system has been described (Nass et al., 1978). In brief, light intensity, from a xenon flash tube, rises to a peak in 40 μs and then decays with a time constant of 250 μs. Measured at the electroplaque, the "standard flash" has a time-integrated flux of 2×10^{17} photons/cm^2 (95 mJ/cm^2) in the 100-nm band centered at 420 nm.

Photochemistry

The photoisomerizable compounds used (Fig. 1) are QBr (bromide salt) and Bis-Q (diiodide salt). Both compounds have the photochemical characteristics of typical azobenzene derivatives; they display nearly identical absorption spectra and photoisomerization properties. If a solution of Bis-Q or QBr is exposed to light of wavelength 300–500 nm, a photostationary state (PSS) is eventually reached. The *cis/trans* ratio of the PSS, although independent of light intensity, irradiation time, and the initial isomeric composition of the solutions, depends strongly on the wavelength of irradiation.

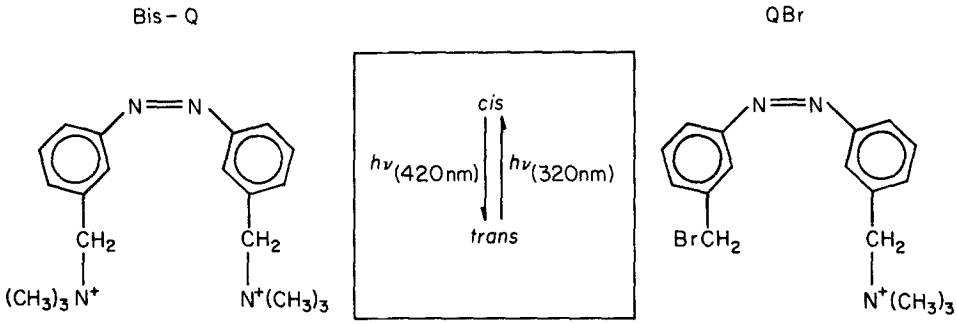

FIGURE 1. Photoisomerizable agonists used in the experiments.

We have recently synthesized pure *cis*- and *trans*-Bis-Q (details to be published elsewhere). There is an isosbestic point at 266 nm ($\epsilon = 4.2 \times 10^3$ liters/mol·cm). The pure *trans* configuration has an absorption peak at 318 nm and $\epsilon_{320}/\epsilon_{266} = 4.9$. For the pure *cis* configuration, $\epsilon_{320}/\epsilon_{266} = 0.53$, and the absorption peak is at 430 nm (Lester et al., 1980). In mixtures of the isomers, the *cis/trans* ratio is determined by linear interpolation between these two values for $\epsilon_{320}/\epsilon_{266}$.

With the data on the pure isomers, we have performed accurate actinometric calibrations on flashes filtered to produce light of various spectra and intensities. For each filter we define the *cis* → *trans* and *trans* → *cis* isomerization probabilities (K_c and K_t, respectively) as follows. The mole fraction of *trans* compound in the PSS is $K_t/(K_c + K_t)$, and the PSS is approached fractionally as $1\text{-exp}[-n(K_c + K_t)]$, where n is the number of flashes. We find that exposure to the unfiltered standard flash gives a PSS that is 65% *trans* (this differs from the assumption of 85% in the paper by Nass et al., 1978), called the "*trans*-PSS." The intensity of the unfiltered standard flash corresponds to $K_c + K_t = 0.4$ flash^{-1}.

The "*cis*-PSS" is obtained when the standard flash is filtered by either of two "UV

only" filters, Chance OX1 (Ealing Corp., S. Natick, Mass.) and Schott UG11 (Schott Optical Glass Co., Duryea, Pa.). This state is 15% *trans* and $K_c + K_t = 0.03$ flash^{-1}.

Conduct of the Experiments

Cells were initially mounted in the experimental chamber in Ringer's solution (Lester et al., 1975) and were checked for normal resting potentials (more negative than −80 mV) and action potentials (> 130 mV). As in our previous studies, solutions applied thereafter contained tetrodotoxin (0.5 μM) to suppress electrically excitable Na channels and $BaCl_2$ (1–3 mM) to maintain the anomalous rectifier in a linear, low-conductance state.

For experiments with QBr, solutions were also buffered to pH 7.5–8.5 in order to accelerate the reduction of disulfide bonds by dithiothreitol (Cleland, 1964). The following pharmacological manipulations were then performed in pool A (bathing the innervated face) (Karlin and Bartels, 1966; Silman and Karlin, 1969; Bartels et al., 1971).

(1) Reduction of disulfide bonds. Cells were exposed to DTT (3–10 mM) for 5–15 min. There was no effect on membrane conductance.

(2) Wash with Ringer solution. With some cells, carbachol (Carb, 50 μM) was applied at this point and no longer produced conductance increases or depolarizations. In some experiments, step 2 was omitted, the DTT being flushed out by the QBr solution; this did not affect results.

(3) Covalent tethering of QBr. This and subsequent steps were performed under orange light (Kodak ML-2 safelight filters, Eastman Kodak Co., Rochester, N.Y.). Solution containing pure *trans*-QBr was placed in pool A for 5–15 min. The concentration was 0.5 μM for experiments at room temperature and 2.5 μM for experiments at 15° and below. Cells depolarized slowly (2–5 mV/min) and agonist-induced conductances developed.

(4) Wash to remove unbound QBr. Most of the experiments were performed after this step.

RESULTS

Properties of the trans-QBr-Induced Conductance

VOLTAGE SENSITIVITY AND VOLTAGE-JUMP RELAXATIONS For reversible agonists, the equilibrium agonist-induced conductance depends on the membrane voltage, increasing at more negative potentials. After a jump of membrane voltage, the agonist-induced conductance relaxes to a new equilibrium value. The same effect is observed with tethered *trans*-QBr (Fig. 2); as for reversible agonists, voltage-jump relaxations follow an approximately exponential time-course and the reciprocal relaxation time, $1/\tau$, decreases at more negative voltage (Fig. 3). The kinetics of *trans*-QBr relaxations are among the fastest we have measured for agonists; at a given temperature and voltage, $1/\tau$ is roughly twice as great as for 50 μM Carb tested on unreduced cells. Tethered *trans*-QBr and reversible agonists seem to display the same voltage sensitivity of kinetics and equilibria (Fig. 3): the equilibrium agonist-induced conductance changes *e*-fold for a 70–100-mV change in membrane potential.

These data show that even when *trans*-QBr is tethered to a receptor, the receptor-channel complex can undergo voltage-sensitive transitions between closed and open states. A similar conclusion was reached on the basis of

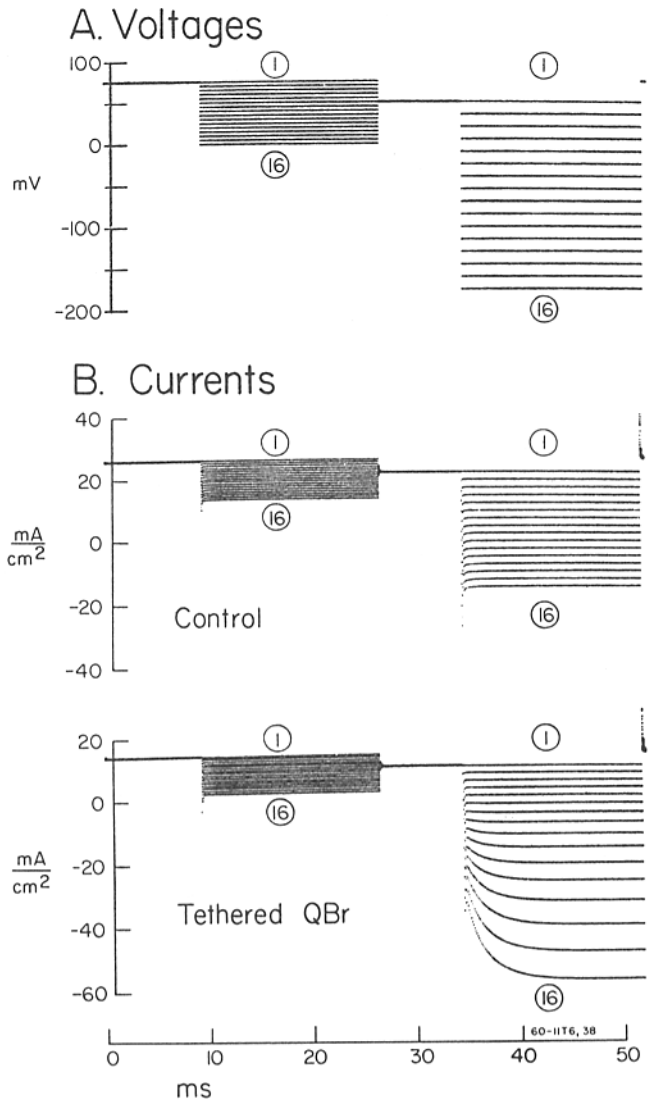

FIGURE 2. Voltage-clamp trials. (A) Voltages during the 16 episodes of a trial. (B) Corresponding currents. Upper panel: untreated cell. Lower panel: same cell after *trans*-QBr has been tethered to receptors and unbound QBr has been washed out (step 4 in Methods). (C) Agonist-induced currents computed from trial in lower panel of B. For each episode, the first half of the trace is multiplied by 3, then subtracted from the second half. Resulting data are passed twice through a recursive digital filter (time constant, 100 μs). Three pairs of cursors are superimposed on traces 8–16. From right to left, these delimit the portions used to calculate the equilibrium agonist-induced current; the exponential approach to equilibrium, and the current before the voltage jump. Upper panel shows semilogarithmic plots of approach to equilibrium, on same time axis; boxes indicate a 10-fold range of current. Temperature, 10°C.

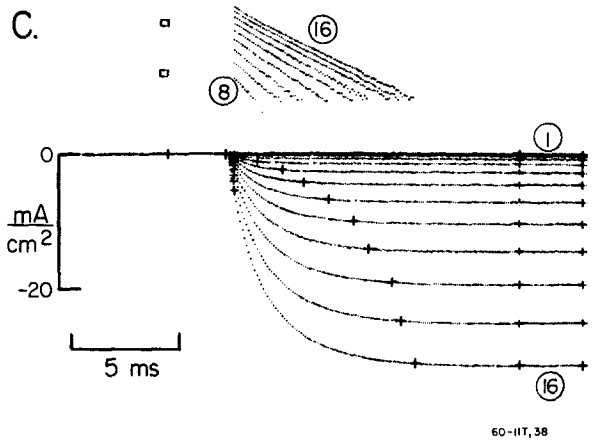

FIGURE 2

fluctuations observed with another tethered agonist (Cox et al., 1979 *a*, *b*). Employing the terminology of Magleby and Stevens (1972 *a*, *b*), we write a simple scheme:

$$(trans\text{-QBr})R \underset{\alpha_{QBr}}{\overset{\beta_{QBr}}{\rightleftharpoons}} (trans\text{-QBr})R^*. \quad (1)$$

The asterisk denotes the open state of the receptor-channel complex. The scheme is written as though each receptor channel is controlled by a single tethered QBr molecule; experimental evidence for this is presented later. For voltage-jump relaxations the rate constant $1/\tau$ is the sum, $\alpha + \beta$. In analyses with reversible agonists, it is found that α increases as the voltage is made more positive, at a rate of about *e*-fold for every 85 mV. The opening rate, β, has little (Magleby and Stevens, 1972 *b*) or no (Sheridan and Lester, 1975, 1977) voltage dependence. We have fit the data for tethered *trans*-QBr with the same assumptions. At -150 mV, $\beta/(\alpha + \beta)$ ranged from 0.2 to 0.6 in different cells; that is, 20–60% of the channels are activated. As with reversible agonists, however, the equilibrium agonist-induced current does seem slightly more voltage sensitive than the simple theory would indicate, possibly because an instantaneous rectification prevents the channels from passing outward current (Sheridan and Lester, 1975, 1977).

EVIDENCE FOR COVALENT BINDING Bartels et al. (1971) noted that extensive washing could not reverse the depolarization due to tethered *trans*-QBr; and we have made similar observations during the present experiments. Indeed, the depolarization and the agonist-induced conductance both increase at step 4, when the unreacted QBr is washed out of the chamber; this is not surprising because *trans*-QBr in solution is a rather weak partial agonist and presumably blocks some receptors. The QBr-induced conductance subsequently decreases with time, the decrement amounting to a factor of about twofold after 2 h. Similar declines, however, occur with repeated bath application of reversible agonist (Koblin and Lester, 1979). The decline could not be accelerated by

extensive washing (150 chamber volumes) with Ringer's solution or with *d*-tubocurarine; an example is the experiment of Fig. 9 below. Although we do not understand the causes of this gradual deterioration in the agonist-induced conductance (cf. Lester, 1978), we therefore feel that it does not arise from dissociation of the tethered *trans*-QBr molecules.

TEMPERATURE SENSITIVITY Fig. 4 compares the temperature dependence of the conductances induced by Carb on unreduced cells and by tethered *trans*-QBr on reduced cells. For both agonists over the range from ~4 to 20°C, the equilibrium agonist-induced conductances show little dependence on temperature ($Q_{10} \sim 1.5$; see also Sheridan and Lester, 1975). Presumably, α and β

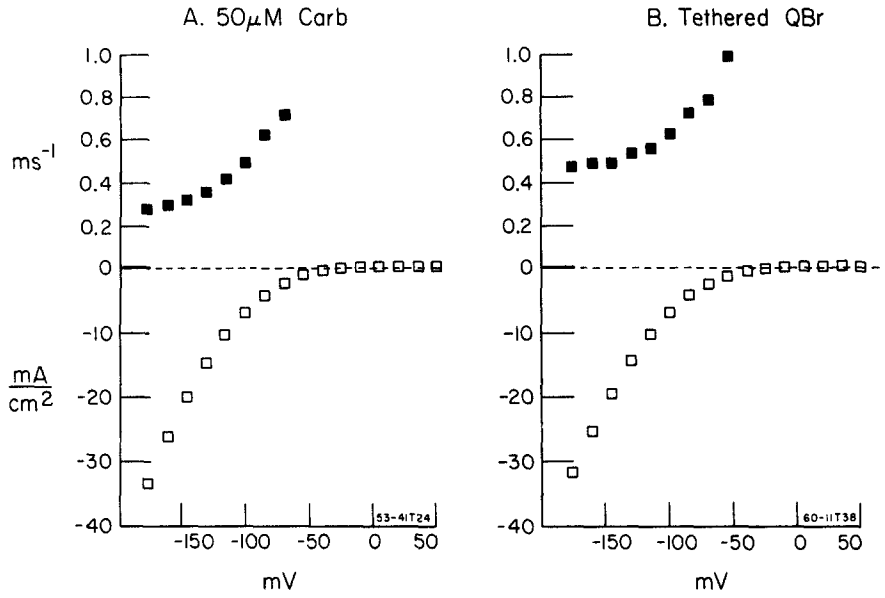

FIGURE 3. Agonist-induced currents (lower panels) and reciprocal time constants $1/\tau$ for voltage-jump relaxations (upper panels). (A) Carb (50 μM); (B) tethered *trans*-QBr (trial of Fig. 2 C). Temperature, 10°C.

have similar temperature sensitivities. On the other hand, the voltage-jump relaxation rate constants do show a high temperature sensitivity; the Q_{10} is between 5 and 6 in this range. This temperature sensitivity is greater than the value of 2.5–3.5 reported for noise and relaxation kinetics with various agonists, including Carb, at muscle fibers (Magleby and Stevens, 1972 *b*; Anderson and Stevens, 1973; Gage and McBurney 1975; Neher and Sakmann, 1975; Dreyer et al., 1976; Fischbach and Lass, 1978) and with decamethonium and Bis-Q at electroplaques (Sheridan and Lester, 1975; Lester and Chang, 1977; Nass et al., 1978). The following additional observations support the surprisingly high Q_{10} measured here with Carb and tethered *trans*-QBr. (*a*) The same Q_{10} was obtained regardless of the direction (up or down) in which

temperature was varied. (*b*) The high temperature dependence is not an artifact of an incorrectly compensated series resistance; in one QBr experiment, we carefully reset this compensation at each temperature and found the same Q_{10}. (*c*) Experiments with Bis-Q were repeated during the present studies. Values of 3–3.5 for the Q_{10} were measured, as reported earlier (Lester and

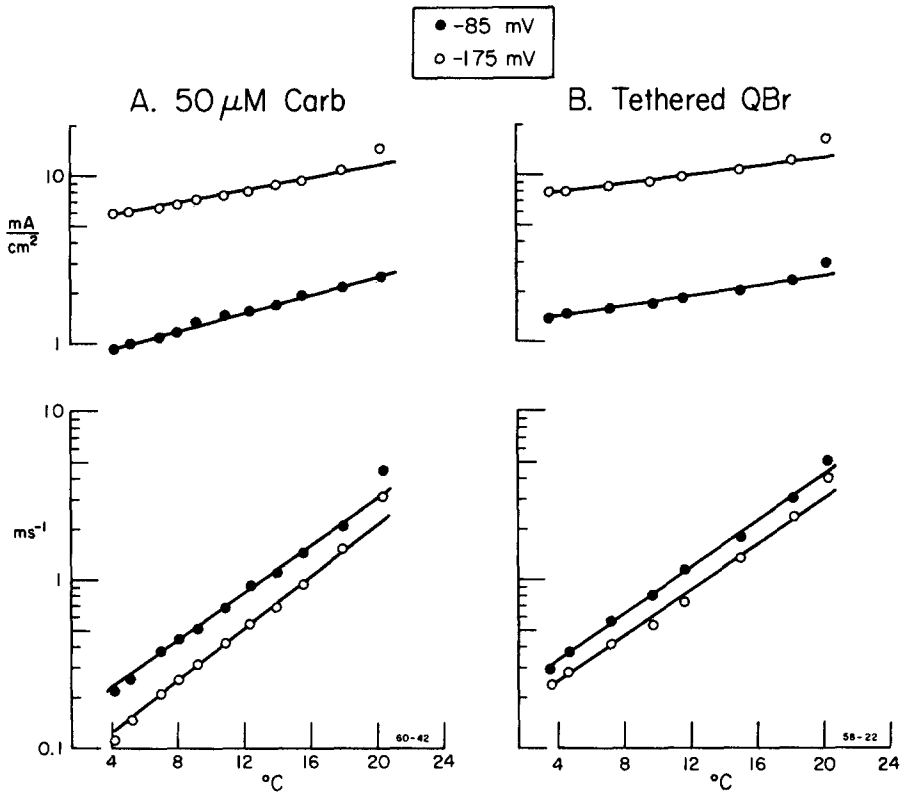

FIGURE 4. Temperature sensitivity. Upper panels: agonist-induced currents at indicated voltage. Lower panels: reciprocal time constants $1/\tau$ for voltage-jump relaxations from +50 mV to the indicated voltage. In each case, all the measurements were made without a solution change. The first trials were taken at room temperature; the preparation was then cooled down over the next 20 min.

Chang, 1977; Nass et al., 1978; and compare Figs. 5 A and 9 A below). We also measured the Q_{10} for acetylcholine and found values between 2.5 and 3.5. It therefore appears that channel kinetics have different temperature dependencies among agonists. This interesting observation deserves further study; for the moment, however, the pertinent observation is that the temperature dependencies for *trans*-QBr fall within the range observed with reversible agonists.

cis- vs. trans-QBr

THE TETHERING REACTION Two observations indicate that *trans*-QBr alkylates receptors much faster than *cis*-QBr. Firstly, although the tethering reaction was usually performed with a solution containing the pure *trans* isomer of QBr, a few experiments were performed with 85% *cis* isomer at the same total QBr concentration (*cis*-PSS solutions). For these cells, a much smaller conductance was measured at step 4, and this could not be increased by light.

Secondly, regardless of the isomeric composition of the solution used for the tethering reaction, the first flash always produced a conductance decrease. This observation is analyzed quantitatively below, but for the moment it implies that the tethered QBr molecules constitute a nearly pure *trans* population before irradiation. A flash then produces some *trans* → *cis* photoisomerizations that decrease the conductance (see next section). Under some conditions, the first flash would have increased the conductance if *cis*-QBr had even a small fraction of the reactivity of the *trans* isomer—for instance, when the solution used for tethering contained 85% *cis*-QBr and the first flash was unfiltered. However, the first flash led to a decrease even under these conditions. We estimate that the alkylation favors the *trans* isomer by at least 15-fold.

THE TETHERED DRUG After step 4, the membrane conductance can be modulated with light. Tethered QBr is converted to the *cis*-PSS by steady exposure to "UV only" light; this partially repolarizes the cell and decreases the agonist-induced conductance. The *trans*-PSS is produced by steady unfiltered light; this causes a depolarization and an increase in agonist-induced currents. The agonist-induced conductance can be cycled over a four- to fivefold range with these procedures. Each half-cycle requires about a 30-s exposure to our light source and we have performed up to six such cycles on a single cell (cf. Fig. 9). After each cycle, the agonist-induced conductance is reduced by ~ 20%. As noted above, this deterioration does not seem specific to QBr: similar decreases are seen with repetitive bath application of reversible agonists (Koblin and Lester, 1979).

Based on these observations, Scheme 1 may be extended:

$$\begin{array}{ccc} (\textit{trans}\text{-QBr})R & \underset{\alpha_{QBr}}{\overset{\beta_{QBr}}{\rightleftharpoons}} & (\textit{trans}\text{-QBr})R^* \\ h\nu \uparrow \downarrow h\nu & & h\nu \uparrow \downarrow h\nu \\ (\textit{cis}\text{-QBr})R & \underset{\alpha_{cis\text{-QBr}}}{\overset{\beta_{cis\text{-QBr}}}{\rightleftharpoons}} & (\textit{cis}\text{-QBr})R^* \end{array} \qquad (2)$$

Because tethered *cis*-QBr is a much poorer agonist than the *trans* isomer, $\beta_{cis\text{-QBr}}/\alpha_{cis\text{-QBr}} \ll 1$.

Light-Flash Relaxations

cis → *trans* PHOTOISOMERIZATIONS In previous studies we examined relations between the kinetics and the equilibrium level of cholinergic excitation

(Sheridan and Lester, 1975, 1977). This information has also been obtained with the soluble photoisomerizable agonist, Bis-Q. In the experiment of Fig. 5 A, a flash of light converts most of the Bis-Q molecules from the inactive *cis* configuration to the *trans* configuration—a potent agonist. As a result, the agonist-induced conductance increases 3.7-fold. Furthermore, the reciprocal relaxation time increases by 70% after the flash, implying that each acetylcholine receptor channel has a higher opening probability in the higher *trans*-Bis-Q concentration. Presumably each receptor is subject to more frequent collisions with agonist molecules.

The analogous experiment can also be performed with the tethered agonist, QBr. In preparation, QBr molecules are tethered to receptors and unreacted QBr is washed away as usual (steps 1–4); the electroplaque is then exposed to steady "UV only" light for ~ 1 min so that at the beginning of the trial the tethered QBr population is in the *cis*-PSS. Just as for the experiment with Bis-Q, during the second episode, a flash converts most of the drug from the *cis* to the *trans* state, and the agonist-induced current increases by a factor of 3.8 (Fig. 5 B). However, the relaxations still have the same reciprocal time constant as before the flash (0.6 ms^{-1}). This result implies that the conductance increases because new receptors enter the active population, yet the newly recruited population has the same opening rate constant as the receptors that had *trans*-QBr bound before the flash. For tethered QBr, the opening rate constant does not depend on collisions between free agonist molecules and receptors; instead, this rate depends on events within the covalently linked agonist-receptor complex.

trans → *cis* PHOTOISOMERIZATIONS With Bis-Q, the conductance decreases when bound *trans* molecules are photoisomerized to the *cis* form; this decrease, called phase 1, occurs so rapidly (~100 μs) that we have not separated its time-course from that of the flash (Nass et al., 1978). Phase 1 occurs because *cis*-Bis-Q is a very poor agonist; it leaves the receptor < 100 μs after being created, and the channel closes.

The conductance also decreases when a "UV-only" flash photoisomerizes tethered *trans*-QBr molecules to the *cis* form (Fig. 6), but the light-flash relaxation is slow enough to be measured with the voltage-clamp circuit. In the cell of Fig. 6, for instance, the light-flash relaxation has a half time of ~ 1 ms. This would be the approximate lifetime of the *cis*-QBr channel, but more detailed studies are vitiated by the small size of the relaxations and by the slow inactivation effects described below (Fig. 8).

THE FIRST FLASH There is, however, a simple case where *trans* → *cis* photoisomerizations give a larger relaxation. The tethered molecules are all in the *trans* configuration if the preparation is kept in the dark during and after the tethering reaction. Therefore, a flash can induce only *trans* → *cis* photoisomerizations (the fraction isomerized depends on wavelength and intensity), and as noted briefly above, the first flash produces a decrease in conductance (Fig. 7). Such relaxations allow an estimate of $\alpha_{cis\text{-}QBr}$. The voltage-jump relaxation occurs when only *trans*-QBr is present. Applying simple relaxation theory, the voltage-jump relaxation rate constant $1/\tau_{V\text{-}jump} = \beta_{QBr} + \alpha_{QBr}$. Because our analyses show that roughly 0.6 of the channels with tethered

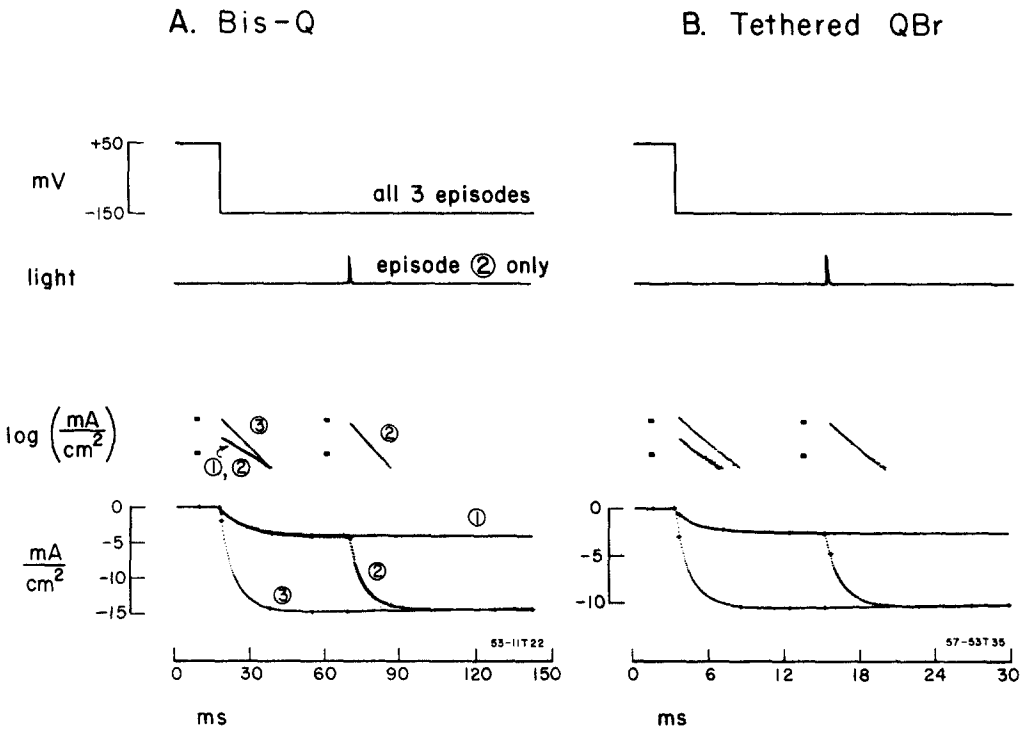

FIGURE 5. Voltage-jump and light-flash relaxations. (A) Bis-Q (400 nM); (B) tethered QBr. Each trial comprises three voltage-clamp episodes, taken at intervals of 0.5 s. Lower traces show agonist-induced currents; leakage and capacitative currents have been subtracted. In A, at the start of the trial, the solution was the *cis*-PSS mixture and contained 60 nM *trans*-Bis-Q; the remaining Bis-Q was in the inactive *cis* form. 15 ms after the start of each episode, the voltage was jumped from +51 to −150 mV. The agonist-induced current increased along an exponential time-course (note semilogarithmic plots which form straight lines; boxes define a 10-fold range). For the first two episodes, these voltage-jump relaxations superimpose; the reciprocal time constant is 0.10 ms^{-1}. About halfway through the second episode, the light flash (four times standard intensity) occurred, increasing the *trans*-Bis-Q concentration to ~ 243 nM (this concentration produces more than half-maximal activation of the agonist-induced conductance). Following this jump of *trans*-Bis-Q concentration, the conductance increased exponentially to a much larger value; the rate constant is 0.17 ms^{-1}. The third voltage-jump relaxation occurred in the higher *trans*-Bis-Q concentration; the rate constant and final conductance equal those for the light-flash relaxation. Temperature 8°C. For the experiment in B, QBr was tethered to receptors, and unbound QBr was washed away (steps 1–4 in Methods). The preparation was then exposed to UV light (see Methods) to convert the tethered QBr molecules to the *cis*-PSS. Thus as in A the trial starts with only 15% of the drug in the *trans* configuration. The voltage-jump and light-flash relaxations all follow an exponential time-course; but the rate constant is the same (0.6 ms^{-1}) in all cases. Temperature, 9°C.

trans-QBr are closed at −150 mV, and because this fraction also equals $\alpha_{QBr}/(\alpha_{QBr} + \beta_{QBr})$, it may be concluded that $\alpha_{QBr} = 0.6(1/\tau_{V\text{-jump}})$. On the other hand, the light-flash relaxation is caused by receptors whose tethered QBr molecules have undergone *trans* → *cis* photoisomerizations. Since *cis*-QBr

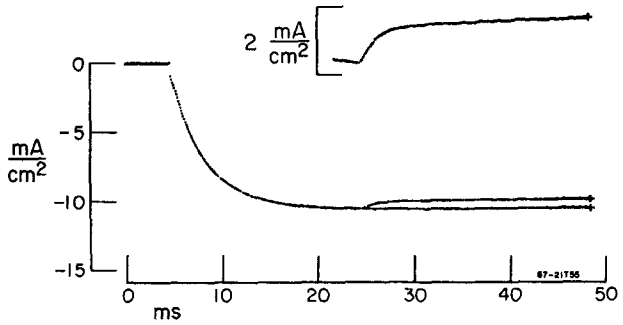

FIGURE 6. The *trans* → *cis* photoisomerization of QBr. Averaged records showing agonist-induced currents from three identical runs, each consisting of two episodes. Trial begins with tethered QBr in the *trans*-PSS. During each episode, the voltage is jumped from +51 to −150 mV. During the second episode of each run, a "UV only" flash occurs (four times standard intensity), leading to a conductance decrease. *Inset* shows light-flash relaxation at higher gain. Temperature, 8°C.

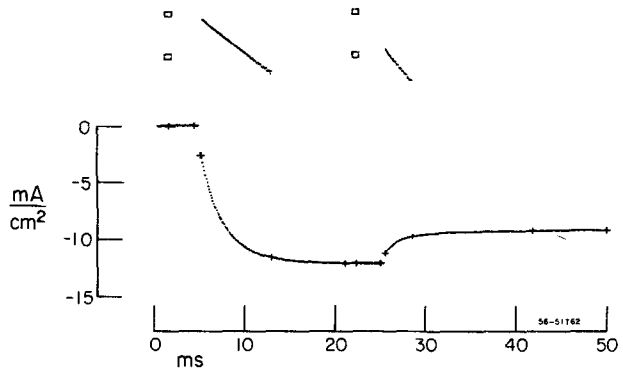

FIGURE 7. *Trans* → *cis* photoisomerization during the first flash applied to an electroplaque with tethered QBr. As in Figs. 5 and 6, the voltage jump from +51 to −150 mV occurs first; an unfiltered flash (four times standard intensity) occurs halfway through the episode. Semilogarithmic plots as in Figs. 2 C and 5. The first 500 μs of each relaxation are not displayed. Data are corrected for the upward sloping base line, estimated by a linear fit to the portion between the rightmost two crosses. Temperature, 11°C.

is a very poor agonist, we assume that the opening rate constant, $\beta_{cis\text{-QBr}}$, is negligibly small; i.e., the *cis*-channels close and never reopen. Therefore $1/\tau_{flash} = \alpha_{cis\text{-QBr}}$. One has $\alpha_{cis\text{-QBr}}/\alpha_{QBr} = (1/\tau_{flash})/(0.6/\tau_{V\text{-jump}})$. In five experiments of this sort, the average ratio $(1/\tau_{flash})/(1/\tau_{V\text{-jump}})$ was 1.52, so

that the *trans*-QBr channel has an average duration ~ 2.5 times that of *cis*-QBr. This ratio is only approximate but it clearly differs from that for the *cis* and *trans* isomers of Bis-Q: the *cis*-Bis-Q channel has a lifetime at least 100 times less than that for *trans*-Bis-Q (Nass et al., 1978).

NO PHASES 3 AND 4 When the reversible photoisomerizable agonist, Bis-Q, is bound to receptors, a flash produces additional relaxations of the conductance on a time scale of 20 ms to several seconds. These relaxations consist of a decrease (phase 3) followed by a slower increase (phase 4) (Nass et al., 1978). In experiments with tethered QBr, we have not observed similar relaxations. There are, indeed, slow decreases of conductance under voltage-clamp conditions (see below), but these occur whether or not a flash has occurred. Nass et al. (1978) considered several possible mechanisms for phases 3 and 4. The present findings support one of the suggestions, namely, that reversibly bound Bis-Q molecules can be released near receptors by a flash and can act as blockers. This interpretation is also supported by experiments with other photoisomerizable azobenzene drugs (Lester et al., 1979 *c*, 1980).

BLOCKADE AT HIGH NEGATIVE POTENTIAL As noted above, the QBr-induced conductance shows a gradual decline on a time scale of ~ 1 h. Here we describe an additional decrease that occurs within a few hundred milliseconds. This phenomenon is most apparent at high negative potentials and can be reversed by stepping the voltage to more positive values (Fig. 8); the rate of this blockade increases with temperature. We have done a few experiments to determine whether the blockade at high negative potential can be influenced by changes in the configuration of the tethered QBr molecules. The rationale here, as in a previous study (Nass et al., 1978), was to use flashes that photoisomerize individual QBr molecules from *cis* to *trans*, and vice versa, but

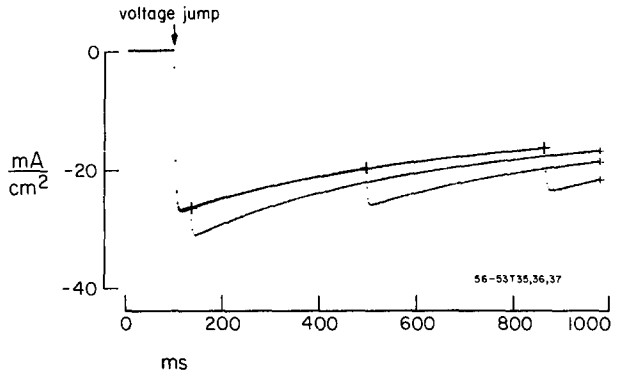

FIGURE 8. Blockade at high negative potential. Agonist-induced currents from three successive voltage-clamp episodes with tethered QBr (*trans*-PSS), showing effect of flashes. Voltage is jumped from +51 to −150 mV at the same time during each episode (arrow). Unfiltered flash (four times standard intensity) also occurs, at a different time during each episode; cross marks position of each trace just before the flash. Note that the agonist-induced current decreases with time after the jump and that the light-flash relaxations increase with time after the jump.

do not change the overall number of *cis*- and *trans*-QBr molecules. This is most easily accomplished by first exposing the preparation to several unfiltered flashes (as in Fig. 5 B) so that the QBr molecules form the *trans*-PSS; the agonist-induced conductance is then measured during further unfiltered flashes (Fig. 8). Experiments of this sort show (*a*) that the blockade at high negative potentials can be partially reversed by such photoisomerizations and (*b*) that as the blocked fraction of the conductance increases, there is an increase in the amount that can be restored by a flash.

To explain these observations, we consider an extension of Scheme 2 in which open channels are selectively able to enter a blocked state (denoted by †):

$$\underset{\alpha_{QBr}}{\overset{\beta_{QBr}}{\rightleftharpoons}} (\textit{trans}\text{-QBr})R^* \overset{\text{slow}}{\rightleftharpoons} (\textit{trans}\text{-QBr})R\dagger. \qquad (3)$$
$$h\nu \uparrow \downarrow h\nu$$

The photoisomerizations proceed with the time-course of the flash (hundreds of microseconds); the β,α-transition equilibrates in a few milliseconds; but the blockade requires hundreds of milliseconds. When the voltage is jumped from a positive to a negative value (as in Fig. 8), receptors with tethered *trans*-QBr molecules undergo a shift from the closed (R) to the open (R^*) state. These receptors then begin a much slower transition to the blocked ($R\dagger$) state, thus depleting the open-closed pool of *trans* receptors. A flash now partially reestablishes the configurational equilibrium among receptors in each of the three functional states. The R, R^* pool therefore experiences a increase in the number of *trans*-QBr molecules; this results in a relaxation to a larger conductance, as in the light-flash relaxations of Fig. 5 B. The blocked pool is shifted toward the *cis* configuration but this causes no immediate change in conductance. (A second-order effect could arise if blocked receptors can be driven back to the R state by this *trans* → *cis* photoisomerization; the conductance would then be increased further by some of the receptors whose tethered QBr molecules underwent an additional *cis* → *trans* isomerization during the flash.)

We do not know the mechanism for this blockade at high negative potentials. Many membrane channels seem vulnerable to "open-channel blockade" by small molecules originally present in either intracellular (Armstrong, 1966) or extracellular solution (Adams, 1975; Neher and Steinbach, 1978; Lester et al., 1979 *c*) or in the membrane phase (Hille, 1977). In the present case there is no known blocking drug present, but open-channel blockade could nevertheless be caused by a component of the Ringer solution or by a tethered QBr molecule. Another possible mechanism is desensitization. At present we have little information on the molecular basis of this effect or on how it can be distinguished from open-channel blockade.

Stoichiometry of the Functional QBr-Receptor Interaction

Agonist-receptor interactions are conventionally studied by measuring the response to varying agonist concentrations. Although one cannot vary the

"concentration" of a covalently bound drug, it is possible to compare (*a*) the fraction of tethered QBr molecules in the *trans* configuration with (*b*) the resulting conductance. We have not measured (*a*) directly, but azobenzene photoisomerizations are not expected to be sensitive to any solvent or binding conditions likely to occur in or near the membrane (Birnbaum and Style, 1954; Zimmerman et al., 1958; Chen and Morawetz, 1976). Therefore, we assume that tethered QBr molecules are photoisomerized independently of each other and with the "rate" constants, K_c and K_t, measured when flashes are applied to solutions of QBr. We made this assumption in previous experiments with reversibily bound Bis-Q (Nass et al., 1978).

We consider three schemes for the stoichiometry of channel activation by tethered QBr (Table I). These schemes differ (*a*) as to how many QBr molecules (one vs. two) are tethered to the receptor associated with each

TABLE I

STOICHIOMETRY OF THE FUNCTIONAL QBr-RECEPTOR COMPLEX

		Theory		
		Scheme A	Scheme B	Scheme C
Possible combinations of tethered QBr molecules		*trans*[a]	(*trans*, *trans*)[a]	(*trans*, *trans*)[a]
			(*cis*, *trans*)[a]	(*cis*, *trans*)
		cis	(*trans*, *cis*)[a]	(*trans*, *cis*)
			(*cis*, *cis*)	(*cis*, *cis*)
Normalized fraction of active receptors, g		t	$2t-t^2$	t^2
t		g_{after}/g_{before}		
Before flash	After flash	Observed	Predicted		
			Scheme A	Scheme B	Scheme C
1.0	0.88*	0.87±0.01 (3 cells)	0.88	0.99	0.78
	0.72‡ (Fig. 7)	0.70±0.03 (4 cells)	0.72	0.92	0.53
0.15	0.31*	2.0, 2.2	2.10	1.91	4.4
	0.55‡ (Fig. 5)	4.0±0.6 (6 cells)	3.66	2.87	13.4

Comparison of theory and experiment for three receptor activation schemes. In scheme A, only one tethered QBr molecule is associated with each channel; in schemes B and C, two tethered QBr molecules are associated with each channel. Superscript *a* denotes active state of the receptor. The parameter t is the fraction of tethered QBr molecules in the *trans* configuration. Values for t are obtained from actinometric calibrations (see Methods) and from the assumption that the photoisomerization probabilities, K_c and K_t, for tethered QBr equal those measured for solutions (see Methods and text). In experiments with $t = 1.0$ before the flash, this was the first flash after the tethering reaction; in experiments with $t = 0.15$ before the flash, the tethered QBr was in the *cis*-PSS. The measurements employed unfiltered flashes at two different intensities, $K_c + K_t = 0.4$ or 1.6 (experiments marked * and ‡, respectively). The measured value g_{after}/g_{before} is the ratio of agonist-induced current just before the flash to the value after the light-flash relaxation, corrected for slow decreases as in Fig. 7. Mean values are given, ± SEM. Voltage, −150 mV; temperature, 7°–11°C.

channel and (*b*) as to how many of these molecules must be in the *trans* configuration to activate the channel. On the assumption that tethered *cis*-QBr is not an agonist at all, each scheme predicts a different relation between the fraction of molecules in the *trans* configuration and the normalized conductance. The predictions were tested under conditions chosen to minimize distortion by the slow decrease. The data (Table I) clearly fit the simplest model best: a single tethered QBr molecule determines whether the receptor is active. Apparently the 250,000-dalton monomer is the functional unit of receptor activation for tethered QBr.

This result is surprising because in studies with reversible agonists, dose-response data generally imply that the open state of the channel is much more likely to be associated with the binding of two agonist molecules than with the binding of just one. We asked whether the DTT reduction alone, or the subsequent tethering of QBr, causes this change in functional stoichiometry. This point could in principle be tested with dose-response studies; previous experiments with Carb suggested that the Hill coefficient changed from roughly 2 in the normal cell to about 1 after DTT reduction (Karlin, 1967; Podleski et al., 1969). However, one would prefer to use data for an agonist that produces greater activation with reduced receptors than with normal receptors to insure that responses are not substantially distorted by a possible small fraction of unreduced receptors. Carbachol, Bis-Q, and acetylcholine all showed substantially smaller activity on DTT-treated cells than on normal cells; hexamethonium gave only small and variable effects. However, we confirmed previous observations (Karlin, 1969; Podleski et al., 1969) that decamethonium (Deca) displays a severalfold-higher apparent affinity for the receptor immediately after DTT treatment. Unfortunately, as also observed in the previous studies, this enhancement was transitory, disappearing over 30 min or so, although the response to Carb was premanently reduced (Karlin, 1969). Therefore, for one cell we reincubated with 10 mM DTT before each Deca exposure; and for another, 1 mM DTT was present in all solutions after the initial 10-min exposure to 10 mM DTT. In both cells, the agonist-induced currents increased more than linearly with [Deca] for concentrations $< 1\ \mu M$. The data are consistent with a power law with an exponent of 1.5–2, as seen for untreated electroplaques (Lester et al., 1975). In view of the difficulties mentioned, these studies must be regarded as preliminary, but it seems that in electroplaques as in muscle, DTT treatment does not change the Hill coefficient of the dose-response curve for agonists (Rang and Ritter, 1971; Ben-Haim et al., 1975). Therefore, the altered stoichiometry probably arises from the covalent attachment of QBr, not simply from the reduction of disulfide bonds.

It may further be noticed that in Fig. 5 A the flash increases the agonist-induced conductance by the same ratio with the reversible agonist, Bis-Q, as with tethered QBr. This does not, however, imply that a single Bis-Q molecule can activate a receptor. The experiment of Fig. 5 A occurred in a linear region of the dose-response relation for Bis-Q; the entire relation is sigmoid with a nonlinear start at low concentration (Lester et al., 1980).

Blocking Drugs

EFFECT OF CURARE We compared the effect of *d*-tubocurarine (dTC) on conductances induced by Bis-Q on unreduced cells and by tethered QBr on DTT-treated cells (Fig. 9). The typical competitive blockade is observed with Bis-Q-induced conductances. For a *trans*-Bis-Q concentration of 60 nM, half-maximal inhibition is produced at a dTC concentration of 320 nM; after a flash has increased the *trans*-Bis-Q concentration to ~ 192 nM, the 50% blockade occurs at ~ 560 nM dTC. These results agree well with the observations that dTC has an apparent dissociation constant of 230 nM (Lester et al., 1975), and that at −150 mV Bis-Q has an apparent dissociation

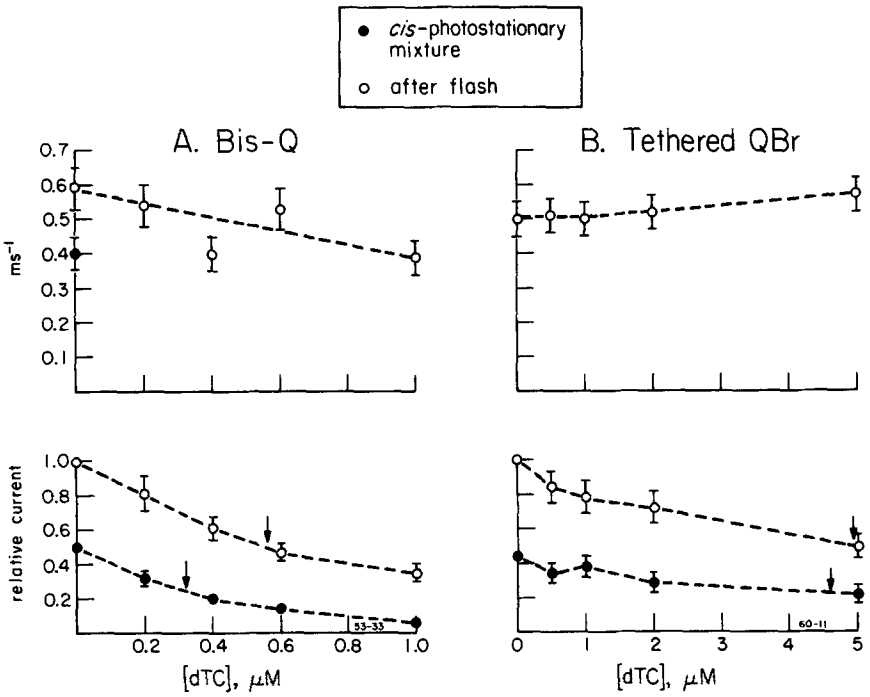

FIGURE 9. Effect of curare. Voltage-clamp trials are like those of Fig. 5, taken in the presence of dTC at various concentrations. In A, fresh Bis-Q solutions (400 nM, *cis*-PSS) were added for each test; but in B tethered QBr was reisomerized to the *cis*-PSS by exposure to UV light after each flash. Unfiltered flashes were delivered at standard intensity (see Methods); flashes jumped the fraction of *trans* isomer from 15% to about 48%. Lower panels give agonist-induced current at −150 mV; to correct for deterioration during the experiment, most dTC measurements were bracketed by controls without dTC. Agonist-induced currents were roughly doubled by the flashes. Vertical arrows show dTC concentration giving 50% inhibition. For 2 μM and 5 μM dTC, QBr-induced currents are peak values before a slow decline (see text). Upper panels give rate constants for voltage-jump relaxations from +51 mV to −150 mV (see Fig. 2). With *cis*-PSS in the presence of dTC, currents were too small for accurate measurements; therefore, kinetic data are shown only for relaxations after the flash. Temperature, 23°C (A), 11°C (B).

constant of about 150 nM.[1] The kinetic data are less clear but suggest a small decrease in the voltage-jump relaxation rate constant as [dTC] increases. Similar effects were noted for dTC and other reversible agonists by Sheridan and Lester (1977).

With tethered QBr, on the other hand, the competitive blockade is much weaker (Silman and Karlin, 1969; Bartels et al., 1971). The dTC concentration must be increased to 5 μM before the agonist-induced currents decrease to half their control value. At 5 μM dTC, the voltage-jump relaxations had a slightly greater rate constant. At voltages more negative than −130 mV, these relaxations were followed by a slower decrease in conductance (time constant, 100–200 ms); this effect is much larger than the slow decrease seen in the absence of dTC. These effects of high [dTC] and high negative potential have also been seen with reversibly bound agonists (Manalis, 1977; Katz and Miledi, 1978; Colquhoun et al., 1979) and can be attributed to "open-channel blockade" by dTC.

As with the experiments on functional stoichiometry, we asked whether the DTT reduction alone, or the subsequent tethering of QBr, caused this change in the effectiveness of curare. Again, decamethonium was the test agonist on DTT-treated cells. In such cells, the decamethonium-induced conductance was still reduced by about twofold in 0.2 μM dTC and by about fivefold in 1 μM dTC. Thus, for at least one reversible agonist, dTC has roughly the same blocking potency before and after DTT treatment. Indeed, Rang and Ritter (1971) report that dTC is actually a more effective blocker after DTT treatment of chick muscle. Our measurements lack the precision to address this point in electroplaques, but it seems clear that the relatively low potency of dTC against tethered QBr arises from the covalent attachment, not simply from the reduction of disulfide bonds.

In terms of Scheme 1, the competitive antagonist dTC does not seem capable of stabilizing the state (or states) denoted by (*trans*-QBr)R. There is a complementary situation: when blocking agents such as MPTA and MBTA are tethered to the sulphur atom, agonists cannot activate the receptor (Karlin and Winnik, 1968; Karlin, 1969).

EFFECTS OF QX-222 This drug is believed to act primarily by prematurely terminating open channels (Steinbach, 1968 *a*, *b*; Beam, 1976 *a*, *b*; Ruff, 1977; Neher and Steinbach, 1978; Koblin and Lester, 1979). At equilibrium, QX-222 had roughly the same effect on the equilibrium conductance induced by Bis-Q at normal cells and by tethered *trans*-QBr. For instance, at 25 μM QX-222 and −150 mV, agonist-induced currents were reduced to one-third of the control values. The relaxations had at least two exponential components, as expected from previous experiments on QX-222 and other suspected "open-channel blockers" (Adams, 1977; Marty, 1978; Koblin and Lester, 1979).

DISCUSSION

A major finding of our work is the close similarity between the conductance induced by tethered *trans*-QBr molecules and that induced by reversibly

[1] Lester, H. A., and M. M. Nass. Unpublished observations.

bound agonists. This similarity includes both equilibrium and kinetic properties and extends to effects both of voltage and temperature. Furthermore, photoisomerizations of tethered QBr affect the conductance just as predicted from the response to the structurally similar, reversibly bound agonist *trans*-Bis-Q, suggesting that tethered QBr and reversible agonists fulfill the same structural requirements for receptor activation (see also Silman and Karlin, 1969). Much current research is addressed to identifying the molecular nature of the events that limit the rate of the transitions between the open and closed states of acetylcholine receptor channels. The similarities just noted suggest that the rate-limiting event, whatever its identity, remains the same for tethered QBr and for reversibly bound agonists. Because QBr is tethered near the agonist binding site but is not covalently bound at this site, the tethering reaction does not complete the agonist-receptor binding step; for instance, the channel might remain closed until the cationic head of QBr moves into a negative subsite on the receptor (Silman and Karlin, 1969). It is clear, however, that tethered QBr molecules do not participate in an initial, diffusion-limited encounter with receptors. One can therefore rule out that the rate-limiting step is the initial encounter between agonist and receptor molecules. Denoting the reversible agonist molecule by A and the initial encounter by $A + R \rightleftharpoons AR$, one may write:

$$2A + R \rightleftharpoons A + AR \rightleftharpoons A_2R \underset{\alpha_A}{\overset{\beta_A}{\rightleftharpoons}} A_2R^*. \tag{4}$$

del Castillo and Katz (1957) first proposed that channel activation required a definite step beyond the mere presence of agonist molecules at or near the receptor binding site. Magleby and Stevens (1972 *a*, *b*) formalized Scheme 4 and assumed that the additional step (governed by β_A and α_A) was the slowest. The QBr experiments now provide definite evidence for this view.

In previous reports of experiments with reversible agonists (Sheridan and Lester, 1975, 1977), it was pointed out that another approach can be used to measure the rate of any intramolecular steps—such as the β,α-transition—necessary to open the channel. This tactic consists of testing whether relaxation rate constants $1/\tau$ level off at high agonist concentrations where the forward binding reaction becomes so fast that it no longer limits the rate of channel opening; if $1/\tau$ does approach an asymptotic value, this rate constant is $\beta + \alpha$. In our previous experiments, this approach was vitiated by the rapid desensitization that accompanies exposure to high agonist concentrations. In experiments on frog nerve-muscle synapses, Sakmann and Adams (1979) have now shown that desensitization does not affect receptor kinetics and $1/\tau$ does level off at high [agonist]. These data also constitute strong evidence for the view that the β, α-transition is the rate-limiting step in Scheme 4.

Molecular Nature of the β,α-Transition

Neither the experiments of Sakmann and Adams (1979) nor those of the present study provide direct confirmation of the specific hypothesis that the β,α-transition is a conformational change involving the receptor protein or

channel (Magleby and Stevens, 1972 *a*, *b*). We can at present only guess at the molecular description of this transition with either reversibly bound or tethered agonists. For tethered agonists, as noted above, Silman and Karlin (1969) and Cox et al. (1979 *a*, *b*) suggested that the agonist's cationic head group can move in and out of a negative subsite on the receptor. The major evidence for this view was that dTC at high concentrations can block the conductance induced by tethered agonists. However, the present experiments show that this blockade occurs at the ion channel rather than at the binding site (Manalis, 1977; Katz and Miledi, 1978; Colquhoun et al., 1979), so we have no direct evidence that the cationic head group ever leaves the negative subsite. Such motion remains possible, however, and could constitute the rate-limiting step in channel gating for tethered agonists.

For reversibly bound agonists, structure-activity considerations suggest that the cationic head group is the most tightly interacting part of the agonist molecule. One would instead expect some "flutter" to occur between the receptor and another part of the agonist molecule, perhaps an electron-rich group such as the carbonyl oxygen of acetylcholine itself or the azo nitrogens of *trans*-Bis-Q (Wassermann et al., 1979).

The final possibility is that developed by Magleby and Stevens (1972 *a*, *b*). Very high rate constants would characterize the rearrangements in the agonist-receptor complex described above, and the rate-limiting step would instead involve a conformational change of the protein which constitutes the binding site or the channel.

Binding Rates

The present findings also have an interesting implication for the rate of the complete agonist-receptor binding reaction. If this step were rate-limiting, its bimolecular forward rate constant would be equal to the pseudo-first-order rate constant describing the proportionality between $1/\tau$ and [agonist]. This rate constant is k_{+2} in the model of Sheridan and Lester (1977, Eq. 13). As discussed in detail in that paper, even if the binding step is not rate-limiting, these values are lower bounds for the actual molecular binding rates. The point at hand is that certain doubly charged agonists have impressively high rates. In the experiment of Fig. 5, for instance, the light flash increases the *trans*-Bis-Q concentration by 183 nM; $1/\tau$ increases by 0.07 ms^{-1}. Therefore, the binding rate is at least 3.8×10^8 $M^{-1}s^{-1}$ at 10°C. In experiments at 23°C (i.e., Fig. 9 A), one obtains values of 1.4×10^9 $M^{-1}s^{-1}$. Less direct arguments yield a similar estimate for the rate of dTC binding (Sheridan and Lester, 1977). Such values approach the maximum theoretical values for a drug-receptor reaction limited by the encounter step alone (Burgen, 1966). The estimates are roughly an order of magnitude lower for singly charged ligands, both from voltage-jump relaxation experiments (Sheridan and Lester, 1977) and from stopped-flow experiments on membrane fragments using fluorescent agonists (Heidmann and Changeux, 1979; Jürss et al., 1979). One is led to consider the role of coulombic forces in accelerating the binding reactions at the receptor (Sheridan and Lester, 1977); for example, ligands might accu-

mulate near the membrane because of negative surface charges (Cohen and Van der Kloot, 1978; Van der Kloot and Cohen, 1979). However, ligands also bind very rapidly to soluble acetylcholinesterase (Rosenberry and Neumann, 1977).

Stoichiometry of Receptor Activation by Tethered QBr

Our analysis led to the conclusion that receptor activation is controlled by the configuration of a single tethered QBr molecule. It may be asked whether this result depends artifactually on the assumptions used to compute the predicted ratios for g_{after}/g_{before} in Table I. We therefore abandoned each important assumption in turn and tested the schemes for receptor activation. (*a*) The flash intensity ($K_c + K_t$) was allowed to differ from the values given by the actinometric calibrations; (*b*) the *cis*- and *trans*-PSS were allowed to have isomeric compositions different from those given by the measurements in Methods; or (*c*) tethered QBr was allowed to have nonzero potency as an agonist. None of these alternative hypotheses gave an internally consistent description of the experimental conductance ratios g_{after}/g_{before}. Therefore, scheme A in Table I with the assumptions given stands as the most likely interpretation of the data.

Our results do not, of course, exclude the possibility that two or more QBr molecules can be tethered per channel (although binding data cited below make this unlikely). If so, however, one of these tethered molecules is unique in that its configuration controls activation. Furthermore, the *trans* configuration is a necessary but not sufficient condition for channel activation: there are still fluctuations between the open and closed state; the transition rates, β_{QBr} and α_{QBr}, depend on voltage and on temperature (Scheme 1).

At this point, we do not know the molecular basis for the contrast between the apparent stoichiometry with tethered QBr and with reversible agonists. The difference could appear at one or more levels of receptor organization. (*a*) The agonist-receptor binding reaction might change character. It was recently suggested that channel activation depends at least partially on the interaction between the receptor and a planar, electron-rich part of the agonist molecule (Wassermann et al., 1979). The energy of this interaction might be as strong for a single tethered QBr molecule as for two reversibly bound agonist molecules. (*b*) There might be changed subunit interactions within a single receptor monomer. Each monomer can bind two molecules of elapid α-toxin, but in fish receptor only one of the two 40,000-dalton subunits can be alkylated by compounds related to QBr (Damle et al., 1978; Moore and Raftery, 1979). It would be interesting to have similar stoichiometric data for the low-affinity binding of reversible agonists which presumably underlies the normal activation of unreduced receptors. (*c*) The interactions between neighboring receptor monomers might change. In connection with possibilities *b* and *c*, it should be noted that sulfhydryl reagents can alter covalent links between subunits and between receptor monomers (Chang and Bock, 1977; Suarez-Isla and Hucho, 1977; Witzemann and Raftery, 1978; Hamilton et al., 1979). Our preliminary results suggest that DTT treatment alone does not

change the stoichiometry of the functional agonist-receptor interaction, but such treatment in combination with the tethering reaction might produce changes of the sort postulated. When more details are known about receptor structure, agonist binding, and channel opening, it will be interesting to compare activation by reversible and tethered agonists.

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