RESPONSE OF ACETYLCHOLINE RECEPTORS TO PHOTOISOMERIZATIONS OF BOUND AGONIST MOLECULES

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ABSTRACT In these experiments, agonist-induced conductance is measured while a sudden perturbation is produced at the agonist-receptor binding site. A voltage-clamped *Electrophorus* electroplaque is exposed to *trans*-Bis-Q, a potent agonist. Some channels are open; these receptors have bound agonist molecules. A light flash isomerizes 3–35% of the *trans*-Bis-Q molecules to their *cis* form, a far poorer agonist. This causes a rapid decrease of membrane conductance (phase 1), followed by a slower increase (phase 2). Phase 1 has the amplitude and wavelength dependence expected if the channel closes within 100 μs after a single bound *trans*-Bis-Q is isomerized, and if the photochemistry of bound Bis-Q resembles that in solution. Therefore, the receptor channel responds rapidly, and with a hundred-fold greater closing rate, after this change in the structure of a bound ligand. Phase 2 (the conductance increase) seems to represent the relaxation back toward equilibrium after phase 1, because (*a*) phase 2 has the same time constant (1–5 ms) as a voltage- or concentration-jump relaxation under identical conditions; and (*b*) phase 2 is smaller if the flash has led to a net decrease in [*trans*-Bis-Q]. Still slower signals follow: phase 3, a decrease of conductance (time constant 5–10 ms); and phase 4, an equal and opposite increase (several seconds). Phase 3 is abolished by curare and does not depend on the history of the membrane voltage. We consider several mechanisms for phases 3 and 4.

INTRODUCTION

Electrophysiological experiments have yielded formal descriptions of opening and closing kinetics for acetylcholine (ACh) receptor channels (1, 2). However, we still lack a molecular description of these rate-limiting steps in channel activation. In experiments described here, we probe these events more directly by manipulating the molecular structure of the drug-receptor complex while monitoring, with electrophysiological techniques, the population of open receptor channels. The perturbations are achieved by photoisomerizing agonist molecules that are reversibly bound to receptors.

The investigations employ 3,3-*bis*[α-(trimethylammonium)methyl]azobenzene (Bis-Q) (3, 4). The *trans* isomer of Bis-Q is a potent agonist at *Electrophorus* electroplaques but few, if any, receptor channels open in the presence of *cis*-Bis-Q. When exposed to light of wavelengths between 300 and 500 nm, Bis-Q molecules in solution

undergo photoisomerization. For the present study, it is important to note that isomerization proceeds in both directions (*cis* → *trans* and *trans* → *cis*) throughout this range; however, the relative probabilities of isomerization depend on wavelength. After prolonged exposure to 320 nm (ultraviolet) light, most Bis-Q molecules are in the *cis* state while 420 nm light produces a predominantly *trans* solution.

We do not yet have access to kinetic measurements on the isomerization of Bis-Q after absorption of a photon. However, in azobenzene and stilbene derivatives, such photochemical isomerizations generally occur in much less than 1 μs at physiological temperatures; there is no evidence for long-lived intermediate states (5). Thus, these structural changes occur instantaneously on the time scale of electrophysiological signals measured in this study (100 μs to several seconds). Even though a photochemical event initiates the relaxations described here, we emphasize that these measurements reflect general properties of the interaction between agonists and the nicotinic receptor. A preliminary report has been published (6).

METHODS

Fig. 1 gives the general plan of the apparatus. Experiments are controlled and analyzed by a minicomputer.

Voltage Clamp

The electrophysiological arrangements have been described in detail (7, 8). In brief, a voltage-clamp trial consists of a series of episodes. Generally a voltage step occurs during each episode and a light flash occurs during one or more of the episodes. Agonist-induced currents are isolated as follows. Electrically excitable Na^+ currents are suppressed with 10^{-6} M tetrodotoxin,

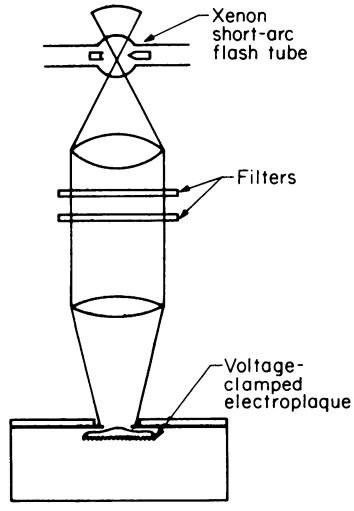

FIGURE 1 Schematic view of apparatus for studying electrophysiological action of photosensitive compounds on single electroplaques from *Electrophorus electricus*. Flash tube was 1 m above preparation. See text.

the anomalous rectifier currents are eliminated with 3 mM Ba^{++}, and passive currents are subtracted. The latter are measured in a range of membrane potentials where there is no agonist-induced current or in the absence of agonist.

A few modifications were made for the present experiments. Currents were passed across the electroplaque between chlorided silver wires or between platinum wires coated with platinum black. Compared with the previously used platinum plates, these electrodes had satisfactorily low resistance and only slightly obscured the light beam.

Some light flashes produced fractionally small changes in voltage-clamp currents (I_m). The following arrangement allowed us to amplify such signals without distortion by capacitive coupling. A voltage proportional to I_m was led to the inverting input of a differential amplifier (gain of 5) as well as to the input of a track-and-hold circuit; the output of the latter circuit was led to the inverting input of the amplifier. The tracked signal was held, starting a few milliseconds before the flash and for the remainder of the episode. The computer received both the original I_m signal and the amplified version with zeroed base line (see Fig. 4*b*).

Optical Arrangements

The flash tube, lamp housing, and trigger circuit were respectively the models 35S, 71, and 99 manufactured by Chadwick-Helmuth Co., Inc., Monrovia, Calif. The final element of the condenser (represented by the top lens in Fig. 1) served as the field aperture in a Koehler-type illumination system. For the "standard flash" this aperture was imaged onto a Mylar sheet containing the window (1 × 3 mm), which exposed the electroplaque's innervated face. The spot had a diameter of 7 mm and nearly filled the upper chamber (pool A). Usually the discharge was delivered from a bank of electrolytic capacitors (500 μF total) charged to 900 V.

In some experiments, we used an additional lens to increase the intensity at the electroplaque by a factor of 4.2 over that of the standard flash (photodiode measurement; see below). To insure that this smaller spot uniformly illuminated the exposed portion of the innervated face, we used a circular window with a diameter of only 1.2 mm.

Actinometric Calibrations

Cis photoequilibrium solutions of Bis-Q were produced by exposure to a 4-W, long-wave ultraviolet (UV) lamp (Pen-Ray 11SC-1L, Ultra-Violet Products, San Gabriel, Calif.), or to the flash tube filtered through a Chance OX1 filter (called "UV-only" light) (Ealing Corp., South Natick, Mass.). The *trans*-equilibrium state was produced with the unfiltered flash or with an in-

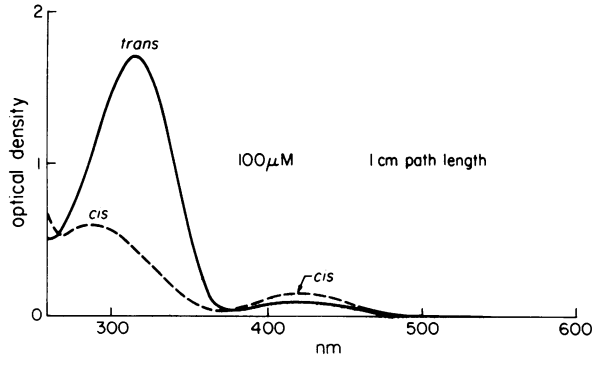

FIGURE 2 Absorption spectra of Bis-Q *cis*- and *trans*- photostationary states. See text for details.

candescent bulb. Fig. 2 presents absorption spectra of the photoequilibrium states. By measuring absorbance at 320 nm it was established that the photoequilibrium states are approached exponentially (with the number of flashes; see below) and with complete reversibility (J. Weissberg and H. A. Lester, unpublished). Both isomers appear to be thermally stable for at least 1 day at room temperature.

Let the concentrations of *cis* and *trans* isomers be C and T, respectively. We now define two "rate" constants, k_C and k_T, by the following statements: (*a*) the photoequilibrium state has the composition $C(\infty)/T(\infty) = k_T/k_C$ and (*b*) the approach to this state is described by $\exp[-(k_T + k_C)n]$, where n is the number of flashes. We measured k_C and k_T as follows.

Solutions of Bis-Q (20 μM) were exposed to flashes in the experimental chamber, optical densities were then measured at 320 nm in a spectrophotometer. For the calibrations the standard flash was attenuated by calibrated neutral density filters, if necessary, so that $k_C + k_T$ equaled 0.015–0.03 flash^{-1}, and 10 flashes were given; solutions were stirred between flashes. This procedure allowed us to minimize variations from one flash to the next and among different regions of the chamber. As the optical density of the solution in the chamber never exceeded 0.15 at any wavelength, no corrections were applied for absorption of the beam. The measurements showed that (*a*) with the unfiltered standard flash, the *trans*-equilibrium state was approached at a rate $k_T + k_C = 0.59$ flash^{-1}; and (*b*) with the standard flash filtered to give UV-only light, the *cis*-equilibrium state was approached at a rate $k_T + k_C = 0.03$ flash^{-1}.

Unfortunately, we do not know $C(\infty)/T(\infty)$ since we have not yet isolated a pure *cis* isomer or *trans* isomer of Bis-Q for determination of physical properties. Most work on azobenzene photochemistry has shown that the photoequilibrium states are about 85% pure (9–11; B. F. Erlanger and N. H. Wasserman, personal communication) and we have adopted these values for calculating k_C/k_T.

These measurements and assumptions yield a value for k_C of 0.50 flash^{-1} with the unfiltered standard flash. This value may be converted to photon flux. Consider the band of wavelengths 100 nm wide centered at 420 nm, the absorption maximum of *cis*-Bis-Q. Most of the *cis* → *trans* isomerization was apparently caused by light in this region since (*a*) k_C was only slightly smaller when the UV light was removed with a Chance OY10 ("No-UV") filter; (*b*) k_C was nearly zero with a yellow Schott glass filter that absorbed light of wavelengths less than 520 nm. The 420 nm peak has a width of 74 nm at half-maximal absorption. If the light has a uniform spectral density, it may be calculated that 75% of the *cis* → *trans* isomerizations are caused by photons with wavelengths in the 100 nm band of interest; as the optical system was inefficient in the UV, the true figure was probably about 85%. The average molar absorptivity $\bar{\epsilon}'$ (defined using natural logarithms and concentrations in moles per cubic centimeter) is 2.35×10^6 over this band. Zimmerman et al. (10) measured a quantum yield for *cis* → *trans* photoisomerization, Φ_T, of 0.55 in the band of interest. Let us now assume that only the intensity, not the spectral distribution, of the flashlamp changes with time (at least for the wavelengths where photons are absorbed by Bis-Q). We may then calculate the time integral of intensity I for a given flash:

$$\int_t I\mathrm{d}t = 0.85\, k_C/\Phi_C\bar{\epsilon}' = 2.0 \times 10^{17}\ \text{photons/cm}^2, \qquad (1)$$

in the 100-nm band centered at 420 nm. Since the spot area was 0.38 cm^2, the flash delivered 7.6×10^{16} photons to the experimental chamber.

The standard flash was also measured with a photodiode (PIN 10DB/541, United Detector Technology, Inc., Santa Monica, Calif.) placed at the chamber. The following calibrated filters were used: 420 nm interference, heat-reflecting interference ("No-IR"), and neutral density. The flash rose to its peak in about 40 μs and decayed with a time constant of 250 μs. Again

assuming uniform photon flux in the band from 370 to 470 nm, the measurements gave a value of 8.4×10^{16} photons (40 mJ) in this band for the unfiltered flash, in satisfactory agreement with the actinometric results.

Temperature Changes

These were measured with a Yellow Springs model 520 thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio). The standard flash caused a temperature rise of less than 0.1°C and of roughly half this amount when the beam was passed through the no-IR filter.

RESULTS

Survey of Relaxations

The records in Fig. 3 provide a general survey of the signals we have observed. The *cis*-photoequilibrium solution of Bis-Q (400 nM) was added to the upper chamber about 1 min before the voltage-clamp trial. In each episode both a voltage jump and, about 37 ms later, a light flash occur. On the basis of the measurements and assumptions described in Methods, it is calculated that the *trans*-Bis-Q concentration is initially 60 nM and that the flashes produce "concentration jumps" to 186, 255, and 293 nM *trans*-Bis-Q.

The first voltage-jump relaxation, in the *cis*-equilibrium solution, resembles those seen with Bis-Q or other agonists (4, 7, 12–15). The conductance increases along an exponential time-course; the reciprocal time constant is 0.15 ms^{-1}. The first light flash

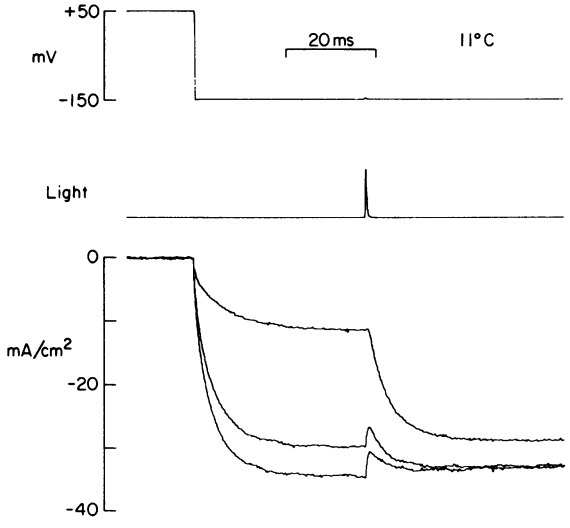

FIGURE 3 Voltage-jump relaxations and "agonist concentration-jump" relaxations in the presence of Bis-Q; see text. Three superimposed episodes at intervals of 20 s. Top trace shows voltage; during each episode, the voltage was jumped from +50 to −150 mV. An unfiltered standard light flash also occurred later in each episode and was monitored by a phototransistor (middle trace). Bottom traces show agonist-induced currents (see Methods for details, including procedures for subtracting leakage and capacitative currents). Cell 45-42 T22,24,26.

then produces roughly a 2.5-fold increase in the agonist-induced current. Such "concentration-jump" relaxations yield information on dose-response relations; these studies will be described more fully in a later paper. For the moment we note that the concentration-jump relaxation follows an exponential time-course and that its reciprocal time constant equals that of the second voltage-jump relaxation (0.20 ms^{-1}). These data extend to higher concentrations the observation that concentration-jump and voltage-jump relaxations have the same time constant under identical conditions (4), and provide further proof that the reciprocal relaxation times increase with agonist concentration (7, 14).

In Fig. 3 the second flash produces an initial rapid decrease in conductance (phase 1, to be described in detail below), followed by a "concentration-jump" increase like that of the first flash. However, the second concentration-jump relaxation is much smaller than the first. There are at least three reasons for this difference. (*a*) As the *trans*-photoequilibrium state is approached, successive flashes produce successively smaller increases of the *trans*-Bis-Q concentration. (*b*) At this voltage half the available receptor channels are open when the *trans*-Bis-Q concentration is 150 nM; thus the dose-conductance relation has begun to saturate (Nass, Lester, and Krouse. In preparation). (*c*) Flashes also induce a slower, temporary closing of receptor channels (phases 3 and 4, to be described below). Indeed, for the third flash, these effects become so important that at the end of the episode the agonist-induced current is less than before the flash, even though the flash produces a small increase in *trans*-Bis-Q concentration.

The Four Phases

This paper concerns relaxations such as those in the final two episodes of Fig. 3. To simplify the kinetic analysis, many of our experiments were performed with flashes that isomerized individual molecules but led to no macroscopic changes in the concentrations of *cis*- and *trans*-Bis-Q. This was usually accomplished by working with the *trans*-equilibrium solution and exposing the preparation to unfiltered flashes. These conditions have almost been attained by the end of the trial in Fig. 3. Under these circumstances a flash produces a sequence of four alternating conductance changes (Fig. 4). We have named these phases 1 through 4 in order of their appearance. Eventually the agonist-induced conductance returns to its value before the flash.

When the *cis*-equilibrium solution is exposed to UV-only flashes, there is again no macroscopic change in *cis* and *trans* concentrations. With electroplaques tested under these conditions, we also observed four phases with very similar kinetics and relative amplitudes (Fig. 5). However, since the UV-only flash produced fairly small conductance changes, we have only limited quantitative measurements on such data.

These phases are not artifacts. None of the phases are seen with light outside the wavelengths (300–500 nm) where Bis-Q absorbs, or with photostable agonists such as carbachol or suberyldicholine. All four phases are seen as usual in electroplaques treated with methanesulfonyl fluoride to inactivate acetylcholinesterase (8). Flashes have no effects on electrically excitable Na^+ currents (studied in the absence of tetrodotoxin).

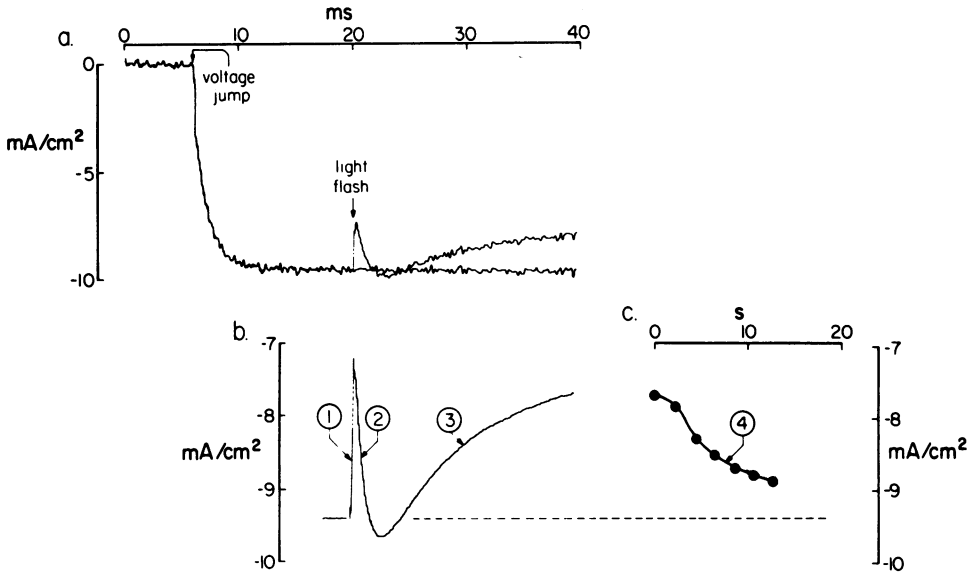

FIGURE 4 The four phases. *Trans*-photoequilibrium solution of Bis-Q (400 nM); temperature 24°C. (*a*) Agonist-induced currents from two episodes 2 s apart. A voltage jump (from +51 to −150 mV) occurred during both episodes; light flash (unfiltered) occurred only during the second episode. (*b*) Amplified trace, 2.4 ms before the flash to 20 ms after flash (see Methods); same time axis as in (*a*). Note phases 1, 2, and 3. (*c*) Time-course for most of phase 4. Additional voltage-clamp episodes, without flashes, occurred at intervals of 2 s after those of panels (*a*) and (*b*). Time axis starts at flash. Points give agonist-induced current at end of each episode. Agonist-induced currents eventually recovered (not shown) to level of dashed line. See text. Cell 47-42 T30, 31.

Phase 1 Results from trans → cis *Photoisomerization of Bound Agonist*

If receptor channels are already opened by *trans*-Bis-Q molecules just before a flash, the flash always produces the rapid decrease in conductance that we call phase 1. We emphasize that phase 1 occurs regardless of any macroscopic change in the *cis*/*trans* concentration ratio produced by the flash. Fig. 5 shows that phase 1 even occurs when a flash is applied to the *cis*-equilibrium solution (this solution contains about 15% *trans*-Bis-Q molecules). (For the experiment of Fig. 3, there was an electrical artifact which mostly obscured the small phase 1 in the *cis*-equilibrium solution.)

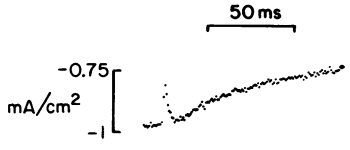

FIGURE 5 Record like that of Fig. 4*b*. *Cis*-photoequilibrium solution of Bis-Q (200 nM). UV-only flash at 4.2 times standard intensity. Trace is the average of four episodes. Temperature 19°C. Cell 51-12 T34.

We have tested the hypothesis that phase 1 results from *trans* → *cis* photoisomerizations of bound agonist molecules. The following assumptions have been made:

(*a*) More than one *trans*-Bis-Q molecule may need to bind to the receptor for channel opening. However we assume that, if one such bound molecule is isomerized to the *cis* isomer, the receptor channel closes.

(*b*) We assume that *cis*-Bis-Q molecules induce a channel closing rate so great that phase 1 reaches completion on the time scale of the flash (50–500 μs). Our clamping circuit has a settling time of 100 μs; therefore this picture would be accurate if *cis*-Bis-Q is such a poor agonist that *cis*-Bis-Q channels have a duration of less than 100 μs and very little probability of reopening.

(*c*) We assume that bound *trans*-Bis-Q molecules have the same "rate" constant for *trans* → *cis* photoisomerization, k_T, as do *trans*-Bis-Q molecules in solution. In photochemical terms the absorption cross-section, ϵ, and the quantum yield for photoisomerization, Φ_T, are not changed by binding (see Methods).

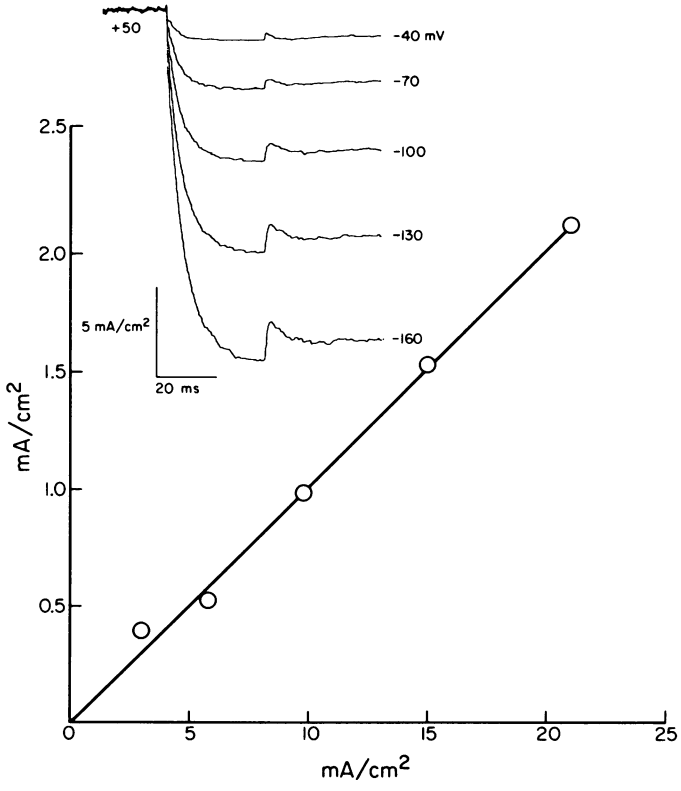

FIGURE 6 Agonist-induced current just before the flash (horizontal axis) compared with amplitude of phase 1 (vertical axis). Equimolar study using *trans*-photoequilibrium solution of Bis-Q (600 nM). For each episode the flash occurred while the voltage was clamped to a different level; see inset. Unfiltered standard light flash; temperature 11°C. Cell 46-12 T 48.

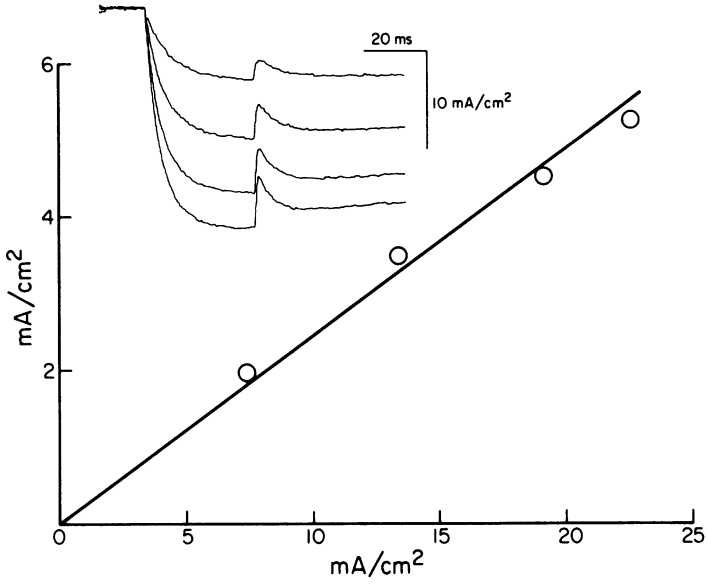

FIGURE 7 Equipotential study complementing Fig. 6. *Trans*-equilibrium solution of Bis-Q (600 nM) was slowly washed into the experimental chamber during the series. In each episode, voltage was jumped from +50 to −150 mV; an unfiltered flash occurred 37 ms later. Temperature 11°C. Cell 46-51.

These assumptions lead one to predict that the amplitude of phase 1 is proportional to the agonist-induced conductance that existed just before the flash. Furthermore, the constant of proportionality should equal the fraction: (molecules in the *trans* state before the flash that undergo at least one photoisomerization)/(molecules in the *trans* state before the flash). This fraction is 1-exp($-k_T$).

The proportionality has been verified with two types of experiments. In the experiment of Fig. 6 we varied the agonist-induced conductance among episodes by varying the membrane voltage (7, 16, 17). In another experiment (Fig. 7) the agonist-induced conductance slowly increased as the *trans*-equilibrium solution was washed into the experimental chamber. Finally, the hypothesis of *trans* → *cis* photoisomerization also predicts the constant of proportionality, both for UV-only and for unfiltered flashes (Fig. 8).

TIME-COURSE OF PHASE 1 Our electrophysiological methods are too slow to resolve the time-course of phase 1. However, using low-resistance electrodes and recording differentially the voltage across the electroplaque, we have determined that phase 1 begins—conductance starts to decrease—less than 80 μs after the flash begins. Voltage-clamp measurements with brief flashes (20 μs decay time constant) have also allowed us to conclude that phase 1 ends and phase 2 starts to dominate—conductance stops decreasing and begins to increase—less than 100 μs after the flash ends. Under these conditions, the amplitude of phase 1 still agrees with the *trans* → *cis* isomeriza-

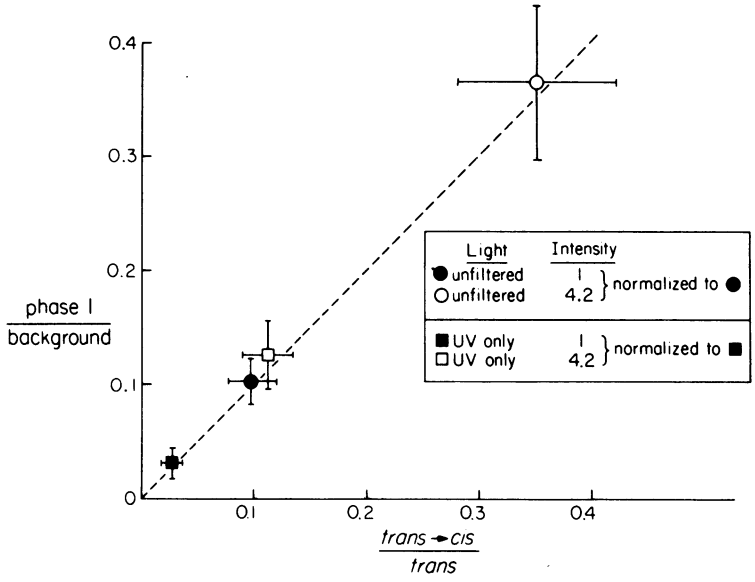

FIGURE 8 Phase 1 versus *trans* → *cis* photoisomerization flux. Horizontal axis is $1 - \exp(-k_T)$; see text. Error bars reflect uncertainties in actinometric calibrations; see Methods. Vertical axis: phase 1 as fraction of agonist-induced conductance just before flash; see Figs. 6 and 7 and text. Error bars give SD (three to six cells per point). *Trans*-photoequilibrium solution of Bis-Q (usually 200 nM); voltage −150 mV; temperatures 10°–25°C. Dashed line at 45° gives prediction of *trans* → *cis* photoisomerization hypothesis (see text).

tion mechanism given above. These observations imply that during phase 1, channels close at a rate in excess of 10 ms^{-1}, even at the lowest temperatures used (10°C).

Phase 2 Is the Relaxation Resulting from the Deficit of Open Channels after the Flash

The unfiltered flash does not affect the concentrations in the *trans*-photostationary solution. If this is also true for the concentration of *trans*-Bis-Q near the receptors (see Discussion), then at the end of phase 1 there are fewer open channels than appropriate for the concentration of agonist. We suggest that during phase 2, the channel population relaxes towards equilibrium again. If this is the case, phase 2 should have an exponential time-course whose time constant equals that of a concentration-jump or voltage-jump relaxation under identical conditions. There are some uncertainties in measuring phase 2, since it is partially obscured by phase 3. We have assumed that phase 3 can be fit as an exponential decrease in membrane conductance superimposed on a sloping base line; and this fit has been subtracted from the voltage-clamp currents (Fig. 9). Phase 2 is usually revealed as an exponential increase in conductance (Fig. 9*b*); at various temperatures its reciprocal time constant agrees well with that for the voltage-jump relaxation (Fig. 10). In Fig. 10 most of the data points lie close to, but not on, the dashed line corresponding to equal time constants. Apparently the dis-

crepancies arise chiefly because of errors in the subtraction of phase 3: where phase 3 has been eliminated by curare, the data points lie on the theoretical line (squares in Fig. 10; see section on phase 3, below). In the *trans*-photoequilibrium solution, voltage jumps immediately after the flash had slightly smaller rates (5–10%) than those immediately preceding it.

ELIMINATION OF PHASE 2 WITH UV-ONLY FLASHES If a flash reduces the concentration of agonist near the receptors, the new equilibrium should consist of fewer open channels than before the flash. In such a case phase 2 might be expected to become smaller than phase 1, to disappear, or even to reverse its sign; the exact results would depend on the size of the concentration jump and on the local shape of the dose-conductance relation. Phase 2 does indeed become less evident with UV-only flashes that reduce the concentration of *trans*-Bis-Q (Fig. 11); but as expected, phase 2 reappears when the *cis*-photoequilibrium solution has been reached (Fig. 5). At present quantitative measurements are vitiated by the relative weakness of the UV-only flash; by the large phase 3; and by the effect described in the next section. However, the results with unfiltered, UV-only, and yellow light agree with the simple hypotheses presented: (*a*) The size of phase 1 depends only on the number of *trans* → *cis* isomerizations. (*b*)

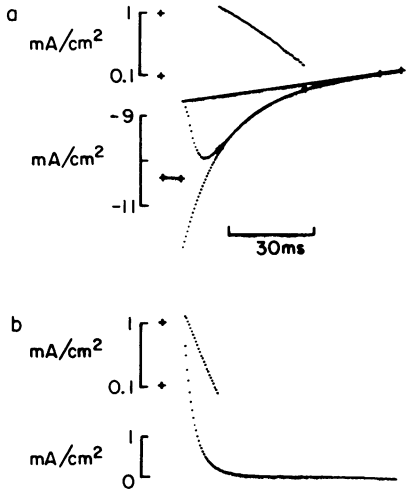

FIGURE 9 Analysis of phases 2 and 3. *Trans*-equilibrium solution of Bis-Q (200 nM), unfiltered flash. Same time axis in all panels. Voltage −150 mV; temperature 13°C. (*a*) Phase 3. Analysis of amplified trace like that of Fig. 4*b*. At start of trace, agonist-induced current was −10.4 mA/cm^2; flash occurred 9 ms later. As a first step in the analysis, a least-squares straight line is fit to final 9 ms of record between two cursors at right. This serves as sloping base line for next step. Beginning at inflection point of the trace, a semilog plot (top panel) is nearly linear. A single exponential is fit (8) to this part of the trace; it superimposes on the data. The exponential plus linear base line are extrapolated to the time of the flash at lower left of this panel. (*b*) Phase 2. Exponential fit to phase 3 is subtracted from the data. Result is shown in bottom panel; semilog plot (top panel) is linear. Cell 47-32 T30.

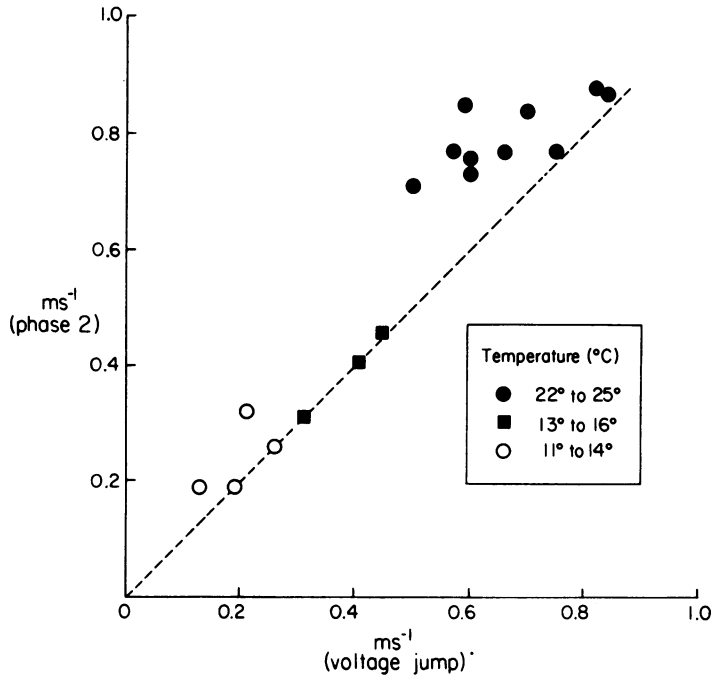

FIGURE 10 Reciprocal time constants for phase 2 (vertical axis, see Fig. 9*b*) and for the voltage-jump relaxation immediately preceding the flash in the same episode (horizontal axis; see 8, 4). Squares refer to data in presence of dTC (2–4 μM). *Trans*-photoequilibrium solution of Bis-Q (usually 200 nM, but 2 μM in presence of dTC); voltage – 150 mV; unfiltered flashes.

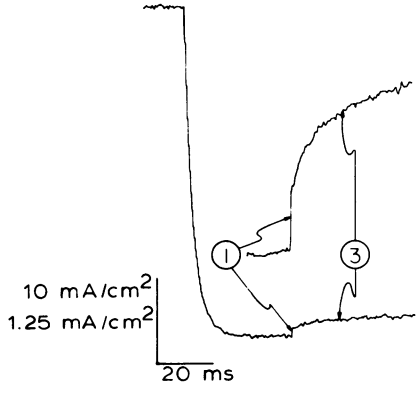

FIGURE 11 Agonist-induced current for a voltage jump from +50 to −150 mV, followed by a UV-only standard flash; average of seven episodes. Trial was started with *trans*-photoequilibrium solution of Bis-Q (600 nM). Same time axis for complete and amplified (inset; see Fig. 4*b*) traces. Note absence of discernible phase 2. Temperature 11°C. Cell 46-21 T35.

Phase 2 depends on the magnitude and sense of the light-induced disequilibrium between the number of open channels and the concentration of *trans*-Bis-Q.

The Transition between Phases 2 and 3

There is a peak of agonist-induced conductance between phases 2 and 3 (see Fig. 3*b*). The temporary maximum of inward (negative) current had a variable amplitude relative to the current just before the flash. Perhaps the solution near the receptors had a different *cis/trans* ratio from that of the photoequilibrium state in the bulk solution, because of buffering by fixed structures in or near the membrane or local filtering of light. More importantly, however, phase 3 itself appears to be reset by each light flash (Fig. 12). We have not been able to investigate this phenomenon in detail because the brightest flashes could be delivered at intervals of no less than 5 s. What seems clear at this point is that the "overshoot" (Fig. 12) never returns the agonist-induced conductance to a level significantly greater than that just preceding the first flash applied to the preparation.

Phases 3 and 4

Phases 3 and 4 disappear almost entirely when *d*-turbocurarine (dTC) is present at concentrations that partially block agonist-induced currents (Fig. 13; see ref. 7). The smaller currents per se do not underlie this action: in the absence of dTC similarly small agonist-induced currents are measured at low *trans*-Bis-Q concentrations, but phases 3 and 4 are still clearly present (Fig. 13*b*, *c*). In preliminary experiments we find that all four phases are still present, although uniformly reduced in size, after treatment with cobra neurotoxin (*Naja naja siamensis* T3), an irreversible postsynaptic blocker (18).

WAVEFORM OF PHASES 3 AND 4 None of our voltage-clamp episodes clearly reveal the transition between phases 3 and 4. In records like those of Figs. 4*b* and 12, the conductance continues to decrease slowly for as much as 150 ms after the flash. At this point phase 3 is already larger than phase 1 (typically by 20–40%). Similar slow

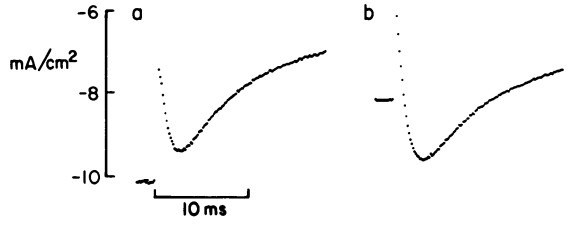

FIGURE 12 A second flash appears to reset phase 3. Amplified records as in Fig. 4*b*. Unfiltered flash occurred during both first (*a*) and second (*b*) episodes, 5 s apart. Note incomplete recovery from phase 3 just before the second flash: the agonist-induced current is smaller than before the first flash (see also Fig. 4*c*). On the other hand, beginning about 3 ms *after* the flash, the conductances are similar in the two episodes. *Trans*-equilibrium solution of Bis-Q (200 nM); voltage −150 mV; unfiltered flashes at about twice standard intensity. Temperature 22°C. Cell 47-51 T51.

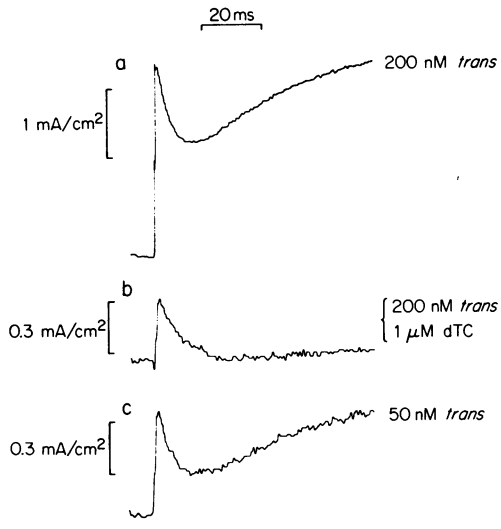

FIGURE 13 Curare abolishes phase 3. Amplified records as in Fig. 4*b*. Unfiltered flashes, 4.2 times standard intensity. Voltage −150 mV. Temperature 11°C. Cell 51-21 T23, 32, 50.

drifts do not usually appear in control episodes where no flash occurs. Phase 3 cannot be characterized as a simple exponential; its time-course sometimes resembles that of a diffusion process.

Despite these quantitative uncertainties, we can offer some preliminary observations based on analyses like that of Fig. 9*a*. In most cases phase 3 has a reciprocal time constant two to four times smaller than that of phase 2 or of the voltage-jump relaxation. The values for phase 3 were 0.090 and 0.1 ms^{-1} (2 cells) at 13°, and 0.24 ± 0.05 ms^{-1} (mean ± SD, 10 cells) at 22–25°. Thus phase 3 clearly proceeds faster at higher temperatures.

PHASE 3 DOES NOT DEPEND ON THE HISTORY OF THE MEMBRANE VOLTAGE. Whether a voltage jump precedes or follows the light flash, phase 3 has the same time-course, relative to the flash, at the final voltage (Fig. 14).

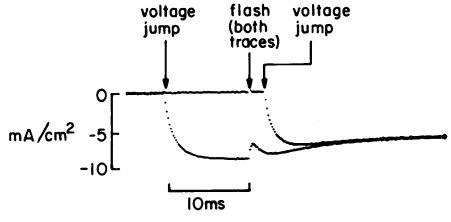

FIGURE 14 Phase 3 depends on the time since the flash and on instantaneous value of the membrane voltage. Agonist-induced currents from two episodes, 36 s apart. Flash occurred at same time in both episodes. Voltage-jump (+50 to −150 mV) preceded the flash in one episode and followed it in the other. *Trans*-equilibrium solution of Bis-Q (200 nM); temperature 23°C. Cell 47-61 T52, 53.

We have incomplete data on the concentration dependence of the rate of phase 3; however, it depends much less strongly on agonist concentration than does either the voltage jump or phase 2. In one cell phase 3 was studied as a function of flash intensity with the *trans*-equilibrium solution of Bis-Q (200 nM). Over a 30-fold intensity range, phase 3 varied in amplitude from 0.15 to 0.43 times the agonist-induced conductance just before the flash; but the reciprocal time constant changed by less than 10% over the series.

PHASE 4 We have relatively little information on phase 4. It has a time-course of seconds (Fig. 4*d*) and it too disappears in the presence of dTC.

DISCUSSION

Which Phases Go Together?

In our view the phases occur in pairs. During phase 2 the receptor population recovers from the process that caused phase 1; and likewise for phases 3 and 4. Phase 4 could also be the recovery from phase 1; phases 2 and 3 would constitute a temporary perturbation, perhaps caused by brief pulse of extra agonist near the receptors. But this seems unlikely for three reasons. (*a*) We describe a condition—curare treatment—that eliminates phases 3 and 4 while leaving 1 and 2 unaffected (Fig. 13). (*b*) Phases 3 and 4 are observed even when the voltage has been set to eliminate or reduce phases 1 and 2 (Fig. 14). (*c*) We have studied certain other photoisomerizable azobenzene derivatives which are not agonists. These compounds do not produce phases 1 and 2; but phenomena similar to phases 3 and 4 still occur (M. M. Nass, H. A. Lester, B. F. Erlanger, and N. H. Wassermann, unpublished observations).

Phases 1 and 2

The cartoon in Fig. 15 summarizes our conception of the molecular basis for phases 1 and 2. We have proposed that phase 1 is caused by a specific event, the *trans* → *cis* photoisomerization of reversibly bound Bis-Q molecules (Fig. 15*a*, *b*). Could channels close simply because an absorbed photon raises a bound *trans*-Bis-Q molecule, or the entire agonist-receptor complex, to an excited state? In the absence of specific information about the agonist-receptor complex, we have assumed that the photochemistry of this complex resembles that of *trans*-Bis-Q in solution. If so, the excited state hypothesis predicts a much larger phase 1 than actually observed. With UV light, only 10% of photons absorbed by *trans*-Bis-Q lead to an isomerization; with visible light the figure is 25% (Φ_T = 0.10 and 0.25, respectively; ref. 10). Therefore with the weaker flashes in Fig. 8 the discrepancies would amount to a factor of 10 for UV-only flashes and a factor of 4 for unfiltered flashes. Of course we cannot rule out the possibility that phase 1 occurs via some other photochemical event whose quantum yield fortuitously matches that of *trans* → *cis* isomerization at the wavelengths tested.

THE BINDING SITE AND CHANNEL ARE CLOSELY COUPLED It is often asked whether channel opening and closing are rate-limited by binding and dissociation of the agonist, or by separate conformational changes that might occur. The results

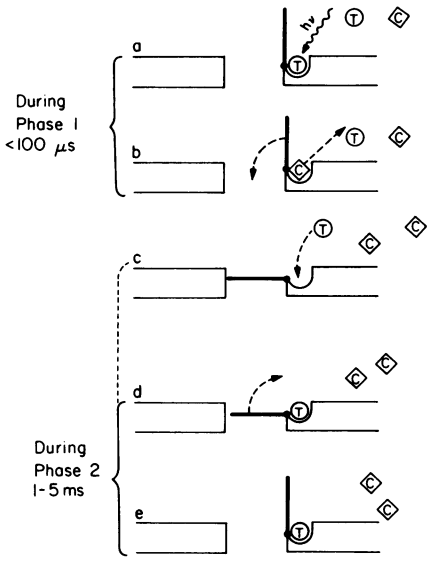

FIGURE 15 Simplified view of events during phases 1 and 2. (*a*) A *trans*-Bis-Q molecule is bound to an acetylcholine receptor and the channel is open. The *trans* molecule will shortly absorb a photon. (*b*) The absorbed photon has led to *trans* → *cis* photoisomerization. Within 100 μs the channel closes as the newly created *cis*-Bis-Q molecule leaves the binding site. (*c*) A *trans*-Bis-Q molecule from the solution binds to the site vacated by the *cis*-Bis-Q molecule. We do not know whether the binding step limits the rate of channel reopening; hence the dashed bracket. (*d*) The channel reopens. (*e*) Channel remains open for the usual lifetime characteristic of *trans*-Bis-Q. See text.

do not resolve this question. Nonetheless the present studies bear more directly on this point than do studies of relaxations resulting from jumps or agonist concentration (4) or of membrane voltage (8, 12–15, 19). A concentration-jump relaxation is caused by perturbations in the nearby solution rather than at the receptors themselves; voltage-jump experiments almost certainly affect the receptor-channel complex, but at an unknown site. By contrast, the present data argue strongly that phase 1 results from an event at the agonist-receptor binding site.

If this is the case, we note that phase 1 has the amplitude predicted by the *trans* → *cis* photoisomerization hypothesis, and that this decrease of conductance attains its full value with the same time-course as the flash intensity (the resolution is defined by the settling time of our clamp, roughly 100 μs). As pointed out in Results, this implies that channels close at a rate of at least 10 ms^{-1} during phase 1, even at 10°C. By contrast, if Bis-Q molecules are not perturbed by a light flash, channels close at a rate of about 0.09 ms^{-1} under the same conditions. Thus a change in agonist structure has led to a 100-fold increase in closing rate. Furthermore, the new closing rate takes effect at the channel with a time delay of less than 80 μs. Other studies have shown that agonist structure influences the opening and closing rates of ACh receptor channels (8, 14, 20–23); of interest here is the large size of this effect and the speed with which infor-

mation moves between the binding site and the channel, if indeed these are separate structures. This information fits well with estimates that receptor channels begin to open less than 150–200 μs after the agonist appears in the synaptic cleft (24, 25).

The details of the photochemical events lead to further conclusions. The studies of Fig. 8 were performed with the *trans*-equilibrium solutions. For the UV-only flashes, most of the photoisomerizations had the direction *trans* → *cis*, and there was a net increase of *cis*-Bis-Q concentration. For the unfiltered flashes there was an even larger rate of *trans* → *cis* isomerizations. However, during these latter flashes, newly created *cis* molecules underwent rapid photoisomerizations back to the *trans* state. During the first 250 μs of the brightest flash, this *cis* → *trans* reisomerization proceeded at a rate approaching 10 ms^{-1} (calculated from k_C and from the time-course of the flash). Suppose that the reisomerization occurred when the *cis*-Bis-Q molecule was still bound to the receptor, with the channel either open or closed. If open, the channel would presumably never close and phase 1 would be smaller, for unfiltered flashes, than predicted by our model. If closed, channels would rapidly reopen, and phase 2 would be more rapid than the voltage jump (26). These arguments suggest that Bis-Q leaves the receptors less than 100 μs after being isomerized to the *cis* state.

There is further evidence for this statement. According to the cartoon, *cis*-Bis-Q molecules must leave the binding site (Fig. 15*b*) before *trans*-Bis-Q molecules can rebind to receptors (Fig. 15*c*). Channels may then reopen (Fig. 15*d*). But with brief (50 μs) flashes, phase 2 dominates (channels reopen) less than 150 μs after the flash begins. Thus at most 150 μs intervenes between channel closure and departure of a *cis*-Bis-Q molecule, though one cannot say which occurs first, even at 10°C. The present conclusion derives from a study of *cis*-Bis-Q, which is a poor agonist; but it may apply to all interactions between ligands and the nicotinic receptor.

The data on phases 1 and 2 all seem consistent with the suggestion that the ACh receptor channel opens as the agonist molecule binds to the receptor (or as the second of two molecules does so) and that the channel closes again as the agonist leaves the receptor (or as either of the two molecules does so) (8, 14, 27).

Phases 3 and 4

We cannot offer a completely satisfactory explanation for phases 3 and 4. One might postulate two general classes of phenomenon: (*a*) after the flash, the receptor or the agonist-receptor complex undergoes intramolecular changes of state; or (*b*) the flash produces pharmacologically active molecules near receptors.

INTRAMOLECULAR CHANGES Certain observations agree with such a mechanism. The time-course of phase 3 depends little on the concentration of Bis-Q or on the number of molecules isomerized (strength of the flash). In many cases phases 1 and 3 have similar amplitudes, suggesting that a receptor participating in phase 1 later participates in phase 3.

One possibility is that the flash converts some receptors to a "desensitized" state, similar to the one obtained after prolonged exposure to high agonist concentrations.

Perhaps Bis-Q molecules, already bound to receptors but not necessarily at the site associated with channel activation, move to a blocking position after absorbing a photon. A subsequent flash would photoisomerize and dislodge some of the blocking molecules, so that the two slow phases would appear to be reset as in Fig. 12. Noncompetitive blockade, presumably at the channel, is seen with many molecules containing both lipid-soluble and amino groups (7, 19, 28–31), and with Bis-Q itself at concentrations greater than 2 μM (unpublished). The original binding would not depend on voltage (Fig. 14), but either the binding or movement would be blocked by curare (Fig. 13). For such a model one would like to know whether the putative blocking molecule is in the *cis* or *trans* conformation before the flash, its conformation after the flash, and where and how tightly it binds both before and during blockade. At present no simple model of this sort explains all the data.

LIGHT-INDUCED CHANGES IN THE CONCENTRATION OF DRUGS NEAR RECEPTORS

In evaluating this possibility we note that high-affinity drugs are buffered by binding to receptors in the synaptic cleft (32–35). Dose-response studies show that *trans*-Bis-Q has an apparent dissociation constant of about 150 nM at −150 mV (M. M. Nass, H. A. Lester, and M. E. Krouse. In preparation). If half the receptors in the synaptic cleft have *trans*-Bis-Q molecules bound when the agonist activity is 150 nM, then the total number of *trans*-Bis-Q molecules per unit volume of the cleft will exceed this activity by at least 100-fold. If our hypothesis for phase 1 is correct, from 10% to 40% of these molecules would be released into the cleft by flashes of the strength used in these studies. By analogy with the equally affine molecule, dTC, buffering by receptors would retain *trans*-Bis-Q molecules in the synaptic cleft for up to 1 s (36). Thus these molecules would have ample opportunity to act as agonists or channel blockers. If dTC binds to the same site, it would reduce the buffering.

There are difficulties with such a mechanism. (*a*) If the receptors are buffering Bis-Q, the extent of buffering should depend on voltage (37); thus phase 3 should depend on the history of the voltage. This is not the case (Fig. 14). (*b*) If acetylcholinesterase is buffering Bis-Q (38), phase 3 should disappear after inactivation of the esterase. This is also not the case.

Phases 3 and 4 deserve further study. In particular one would like to know the pharmacology of the pure *cis* isomer of Bis-Q; and one would like to have comparable experiments with other photoisomerizable drugs.

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NOTE ADDED IN PROOF

We have recently conducted experiments with a pure *cis* isomer of Bis-Q, synthesized by N. H. Wasserman and B. F. Erlanger. At concentrations less than 1 μM, this compound has no effect on receptors.

DISCUSSION

SCHECHTER: We begin with several questions submitted by the referees. Question 1: Does a light flash cause any change in either open or closed receptors other than that mediated by isomerization of Bis-Q?

NASS: Well, none of the phases I've described occurs with photo-stable agonists; that's an important control. Bis-Q itself does not absorb enough energy from the flash to cause a significant temperature jump in the bulk solution. In fact we calculate and measure a change of less than 50 millidegrees per incident light flash. But it is an interesting hypothesis that because of the high receptor concentration in this synaptic cleft, enough Bis-Q accumulates to cause significant local heating when photons are absorbed. This temperature rise would of course be conducted away, but we do not know how rapidly.

SCHECHTER: Question 2: Does *cis*-Bis-Q bind to receptors?

NASS: Well, just recently, working with Norbert Wasserman and Bernard Erlanger at Columbia,