

Functional Stoichiometry at the Nicotinic Receptor

The Photon Cross Section for Phase 1 Corresponds to Two Bis-Q Molecules per Channel

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ABSTRACT These experiments examine changes in the agonist-induced conductance that occur when the agonist-receptor complex is perturbed. Voltage-clamped *Electrophorus* electroplaques are exposed to the photoisomerizable agonist *trans*-Bis-Q. A 1- μ s laser flash photoisomerizes some *trans*-Bis-Q molecules bound to receptors; because the *cis* configuration is not an agonist, receptor channels close within a few hundred microseconds. This effect is called phase 1. We compare (a) the fraction of channels that close during phase 1 with (b) the fraction of *trans*-Bis-Q molecules that undergo *trans* \rightarrow *cis* photoisomerization. Parameter *a* is measured as the fractional diminution in voltage-clamp currents during phase 1. Parameter *b* is measured by changes in the optical spectra of Bis-Q solutions caused by flashes. At low flash intensities, *a* is twice *b*, which shows that the channel can be closed by photoisomerizing either of two bound agonist molecules. Conventional dose-response studies with *trans*-Bis-Q also give a Hill coefficient of two. As a partial control for changes in the photochemistry caused by binding of Bis-Q to receptors, spectral measurements are performed on the photoisomerizable agonist QBr, covalently bound to solubilized acetylcholine receptors from *Torpedo*. The bound and free agonist molecules have the same photoisomerization properties. These results verify the concept that the open state of the acetylcholine receptor channel is much more likely to be associated with the presence of two bound agonist molecules than with a single such molecule.

INTRODUCTION

The nicotinic acetylcholine receptor displays two binding sites for reversible agonists, reversible antagonists, or elapid α -toxin per monomer of molecular

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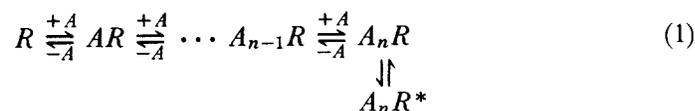
weight 250,000–290,000. Several lines of evidence suggest that the open state of the receptor channel is much more likely to be associated with the presence of agonist molecules at both sites than with a single bound agonist. (a) The agonist-induced conductance is characterized by dose-response relations with a sigmoid shape; the Hill coefficient is near two (Adams, 1975; Lester et al., 1978; Dionne et al., 1978; but see Dreyer et al., 1978). (b) The same Hill coefficient is observed for subsecond measurements of ion flux in receptor-rich vesicles from *Torpedo* electroplaques (Neubig and Cohen, 1980; Cash and Hess, 1980). (c) If one or both of the binding sites is occupied by α -toxin, the channel cannot be activated (Sine and Taylor, 1980).

Can this topic of functional agonist-receptor stoichiometry be addressed more directly, and on the submillisecond time scale appropriate to the acetylcholine-receptor interaction during the growth phase of a miniature postsynaptic potential? Our approach is to examine the consequences of perturbing active agonist-receptor complexes. This experiment exploits the photochemical and pharmacological properties of 3,3'-bis-[α -(trimethylammonium) methyl] azobenzene (Bis-Q) (Bartels et al., 1971; Lester and Chang, 1977). The *trans* configuration of this molecule is at least 100 times more potent as an agonist than the *cis* isomer at *Electrophorus* electroplaques (Krouse et al., 1980). Therefore, when bound *trans*-Bis-Q molecules are photoisomerized to the *cis* configuration, channels close (Nass et al., 1978). The resulting conductance decrease, which we term phase 1, is the fastest known process at acetylcholine receptors: at 10°C it starts within 10 μ s (Krouse et al., 1980) and has a rate constant in excess of 10 ms^{-1} , or ~ 100 times faster than the normal closing rate for channels activated by *trans*-Bis-Q (Nass et al., 1978). For a given flash intensity, a constant fraction of the open receptor channels close during phase 1. This fraction does not depend on the membrane voltage or on the *trans*-Bis-Q concentration (Nass et al., 1978).

The rationale of the present experiment is to measure the fraction of channels that close during phase 1 and to compare this number with the independently measured fraction of molecules that undergo *trans* \rightarrow *cis* photoisomerizations during the flash. If the binding of n agonist molecules constitutes a necessary condition for maintaining the channel in the open state, then the *trans* \rightarrow *cis* photoisomerization of any of the n *trans*-Bis-Q molecules constitutes a sufficient condition to close the channel. For weak flashes, the fraction of channels that close is then n times the fraction of *trans* \rightarrow *cis* photoisomerizations. The dose-response data led us to expect a finding of $n = 2$. This expectation has been confirmed, as reported briefly (Sheridan and Lester, 1981).

THEORY

We begin by presenting the usual model for an acetylcholine receptor channel R , whose open state R^* requires n bound agonist molecules A :



close it. A receptor-channel complex might conceivably undergo the closed \rightarrow open transition with $<n$ bound agonist molecules, then rapidly undergo a transition favoring the binding of additional agonist molecules (cf. Dionne et al., 1978). If so, the experiments should provide information on the relative probabilities of the fully liganded vs. partially liganded open state (see the Discussion).

METHODS

These experiments involve simultaneous electrophysiological and photochemical manipulations on single isolated electroplaques from *Electrophorus electricus*. We have previously given details on the handling of animals, cell mount and physiological chamber, Ringer solutions, voltage-clamp circuitry, subtraction of passive and capacitive currents, and computer control and analysis of experiments (Sheridan and Lester, 1977; Nass et al., 1978; Lester, 1978; Lester et al., 1980).

Voltage Clamp

For these experiments, the major modification was careful optical shielding to avoid photoelectric artifacts in the platinum current electrodes. Pool A, bathing the innervated face, was machined from a piece of amber Plexiglas (2422; Rohm and Haas Co., Philadelphia, PA) that is opaque to the blue flashes. Inlet and outlet tubes were also machined in this piece and a platinum spiral was placed in each tube, separated from pool A by a light baffle. These spirals, connected in parallel, served as the current electrode in pool A. This arrangement gave a higher access resistance than an electrode directly in pool A—600 Ω vs. $<100 \Omega$ —and consequently a slower clamp; in response to voltage jumps, the capacitive transient required $\sim 250 \mu\text{s}$ to settle to 10% of the final value. The electrode in pool B (bathing the noninnervated face) was shielded by black cloth, which also served to prevent reflected light from influencing the measurements.

Optics

Light was produced by a coaxial flashlamp-pumped pulsed dye laser (SLL 625; Candela, Natick, MA) operating at 440 nm with coumarin 450 dye. Flash energy, monitored before and after each experiment with a bolometer, was ~ 100 mJ. The beam was passed through neutral density filters calibrated at 440 nm, then rendered slightly convergent with a 1-diopter lens at a distance of 50 cm from the cell. The beam diameter, measured by burning exposed Polaroid film, was 8 mm.

The experiments depend on accurate knowledge of K_t , the potency of a given flash for *trans* \rightarrow *cis* photoisomerizations of Bis-Q molecules. As in our previous studies, this parameter is measured by exploiting the differences between the optical absorption spectra of the two configurations. The measurements have improved accuracy here because (a) the flashlamp beam has been replaced by a nearly collimated laser beam, and (b) a special cuvette is used. This cuvette has a light path of 10 mm and is mostly filled by a black plastic insert which contains a 5-mm-diam cavity. The cavity contains a solution of Bis-Q (50 μM) and is placed in the position normally occupied by the window that exposes the innervated face of the electroplaque to pool A. Optical densities are measured at 320 nm (the absorption peak for *trans*-Bis-Q) and at 266 nm (the isosbestic point) in a spectrophotometer (Beckman DU, modified by Gilford Instruments, Oberlin, OH) with a suitably masked beam.

A typical calibration series (Table I) begins with the pure *trans* solution. Absorption measurements are made during a series of flashes that eventually produce a predom-

inantly *trans* photostationary state. The cuvette is then exposed to steady ultraviolet (UV) illumination to produce a predominantly *cis* state and another series of measurements is conducted with flashes. This again produces the predominantly *trans* photostationary state. When the approach to the photostationary state is plotted against the number of flashes on semilogarithmic coordinates (Fig. 1), a straight line is obtained and the slope is $K_c + K_t = 0.68 \text{ flash}^{-1}$ (after an increase of 2% to correct for the absorbance of the Bis-Q solution at 440 nm). The fraction of *cis* in this state is 0.34 from the ratio of optical densities at 320 and 266 nm (Lester et al., 1980); since this fraction equals $K_t/(K_c + K_t)$, one has $K_t = 0.23 \text{ flash}^{-1}$. Calibration series of this sort are performed before and after each day's experiments; the measured values for K_t drift by <4% during the ~6-h period. Dimmer flashes are required for some of the electrophysiological measurements. Therefore, neutral density filters are inserted into the beam to reduce the intensity to the range $K_t = 0.01\text{--}0.04 \text{ flash}^{-1}$ (see Fig. 5).

TABLE I
ACTINOMETRIC MEASUREMENT OF FLASH INTENSITY

	A_{266}	A_{320}	$ A_{320} - A_{320}^{\circ} $
Pure <i>trans</i> -Bis-Q (50 μM)	0.211	0.955	0.239
After one flash	0.212	0.836	0.120
After two flashes	0.215	0.779	0.063
After three flashes	0.213	0.747	0.031
After four flashes	0.215	0.731	0.015
After eight flashes (<i>trans</i> -PSS)	0.213	0.718 (A_{320}°)	—
After 10 min UV irradiation (<i>cis</i> -PSS)	0.212	0.353	0.363
After one flash	0.213	0.521	0.195
After two flashes	0.212	0.614	0.102
After three flashes	0.212	0.660	0.056
After four flashes	0.212	0.687	0.029
After eight flashes (<i>trans</i> -PSS)	0.212	0.714 (A_{320}°)	—

The values in the last column are calculated using the average value for A_{320}° from the two series, 0.716.

Time Course of the Photoisomerizations

Our approach assumes that the photochemical events occur much faster than the electrophysiological measurements. This was tested by monitoring the absorbance during the photoisomerizations. A monitoring beam was produced by a mercury vapor lamp (6137; Oriel Corp. of America, Stamford, CT) and a monochromator (DU; Beckman Instruments, Inc., Fullerton, CA) set to 341 nm. This beam passed through a cuvette containing Bis-Q (20 μM) and a 341-nm interference filter and was detected by a 1P28 photomultiplier tube (it would be preferable to monitor at 320 nm but the proper filter was not available). The monitoring beam maintained the Bis-Q in a predominantly *cis* state. The laser flash was introduced at right angles to this beam. The flash itself, measured by removing the interference filter and integrating the signal from the photomultiplier, was complete in $\sim 1 \mu\text{s}$. The absorbance increase had the same time course, with no detectable delay (Fig. 2). No further changes were detected on a longer time scale. These measurements have a temporal resolution of 200–300 ns, so it can be concluded that the photoisomerization is complete within at most several hundred nanoseconds after a Bis-Q molecule absorbs a photon.

QBr Bound to Receptor-rich Membrane Fragments

Membrane fragments rich in acetylcholine receptors were purified from frozen electric organs of *Torpedo californica* (Elliott et al., 1980), extracted at pH 11 at room temperature, and washed twice by centrifugation (25,000 *g*, 30 min) and resuspension in 10 mM Na-phosphate buffer, pH 7.4 (this buffer was used for all subsequent work). Aliquots were assayed for [¹²⁵I]- α -bungarotoxin binding (Blanchard et al., 1979). Membranes were resuspended at $\sim 0.3 \mu\text{M}$ α -bungarotoxin binding sites, incubated with or without $3 \mu\text{M}$ α -bungarotoxin at 23°C for 30 min, washed, and resuspended at the same concentration. Membranes were incubated for 20 min at 23° with 10 mM dithiothreitol, then for an additional 30 min with or without $1 \mu\text{M}$ *trans*-QBr (Bartels et al., 1971; Lester et al., 1980). Membranes were washed three times, resuspended in

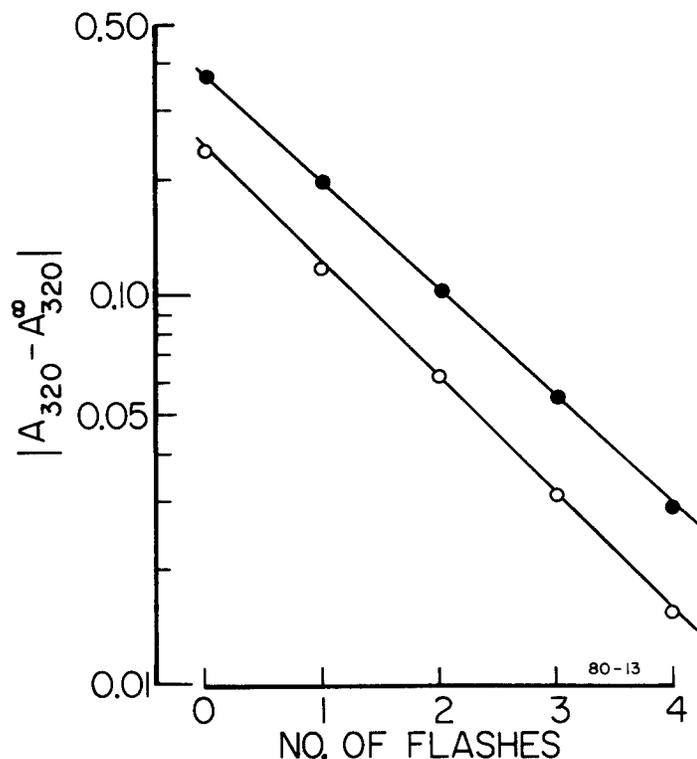


FIGURE 1. Actinometric measurement of $K_c + K_t$. Semilogarithmic plots of the data in Table I. O, series beginning with pure *trans*-Bis-Q; ●, series beginning with the *cis*-photostationary state. See text.

buffer plus 1% Triton X-100 at a concentration of 10–20 μM QBr, and incubated at 4°C for 24 h. The extracts were centrifuged and the supernatant was placed in a standard 1-cm cuvette. The cuvette was exposed to laser flashes as described in the preceding paragraph. Spectra were recorded on a dual-beam scanning spectrophotometer (110; Hitachi, Mountain View, CA). The reference cuvette contained a sample whose processing was identical except for the absence of QBr during one incubation. The optical density at 320 nm was used to determine the QBr concentration, with an aqueous solution of *trans*-QBr as standard.

RESULTS

Dose-Response Studies

The start of the dose-response curve was investigated by measuring agonist-induced currents while electroplaques were exposed to various concentrations of *trans*-Bis-Q. Desensitization does not significantly affect the responses at these low conductance levels (Lester et al., 1975). As expected from previous studies with other agonists, the data yield a slope very near two when plotted on double-logarithmic coordinates (Fig. 3). For technical reasons, the start of the dose-response curve can be studied with greatest sensitivity at high negative potentials; and in the experiment of Fig. 3 at -145 mV, the slope was 2.13. The average value for this slope was 1.92 ± 0.08 (mean \pm SEM, six cells).

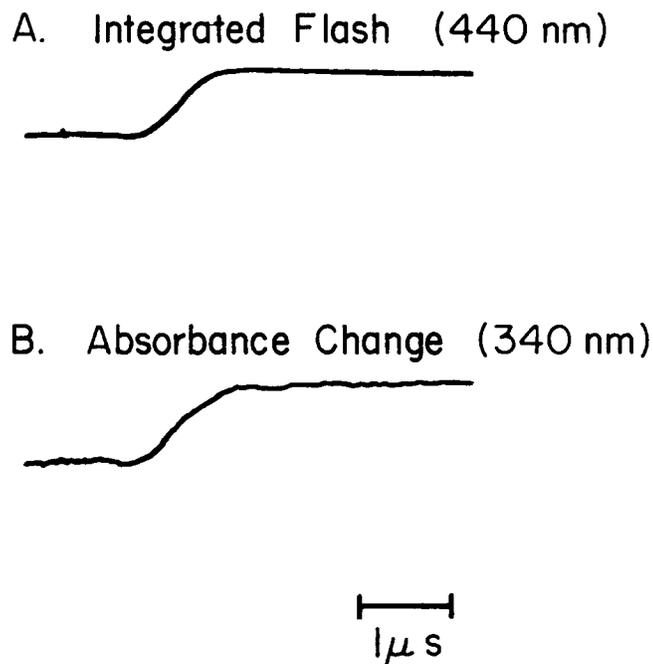


FIGURE 2. Time course of photoisomerizations during a laser flash applied to a cuvette containing Bis-Q solution. The upper trace is the output of a photomultiplier tube, monitoring the flash itself and integrated to show its time course. In the lower trace, the tube measures the intensity of a beam (341 nm) that has traversed the cuvette. There is no detectable difference between the two traces, which shows that photoisomerization is rapid on this time scale.

Cross Section for Phase 1

Phase 1, the deflection labeled ΔI in the experiment of Fig. 4, is a rapid (<1 ms) decrease of agonist-induced current that accompanies *trans* \rightarrow *cis* photo-

isomerizations of *trans*-Bis-Q molecules bound to acetylcholine receptors. The measurement of interest is $\Delta I/I$, the fractional decrease of agonist-induced current represented by phase 1. In Fig. 5, $\Delta I/I$ is plotted vs. K_t , the *trans* \rightarrow *cis* photoisomerization potency, along with lines for nK_t . Clearly the data agree best with $n = 2$; and a similar result was found for all cells tested (Table II).

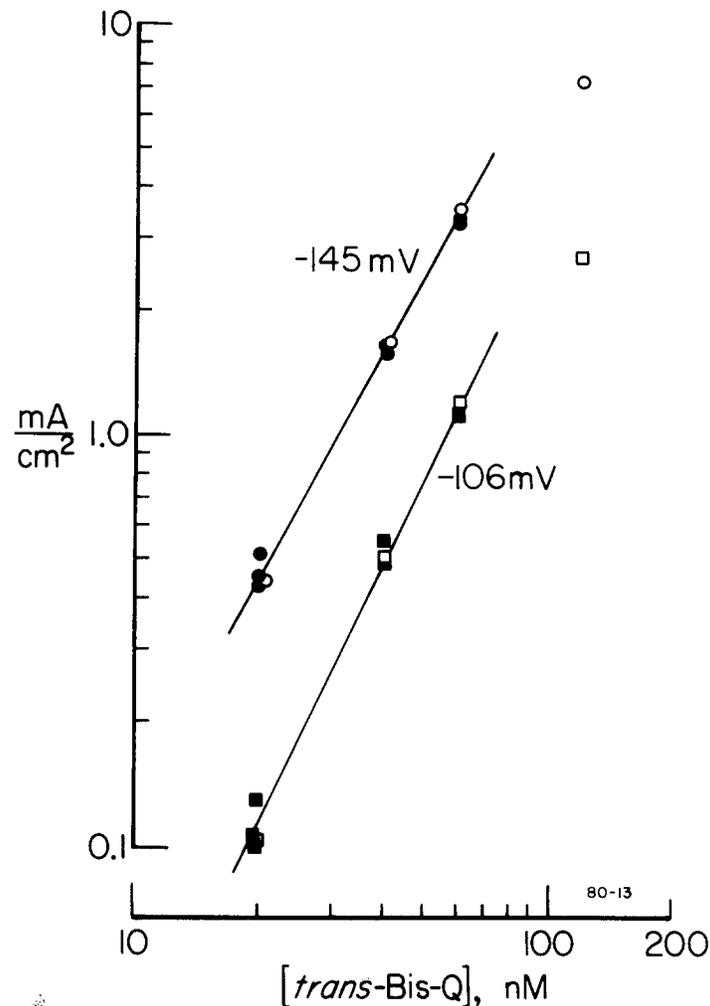
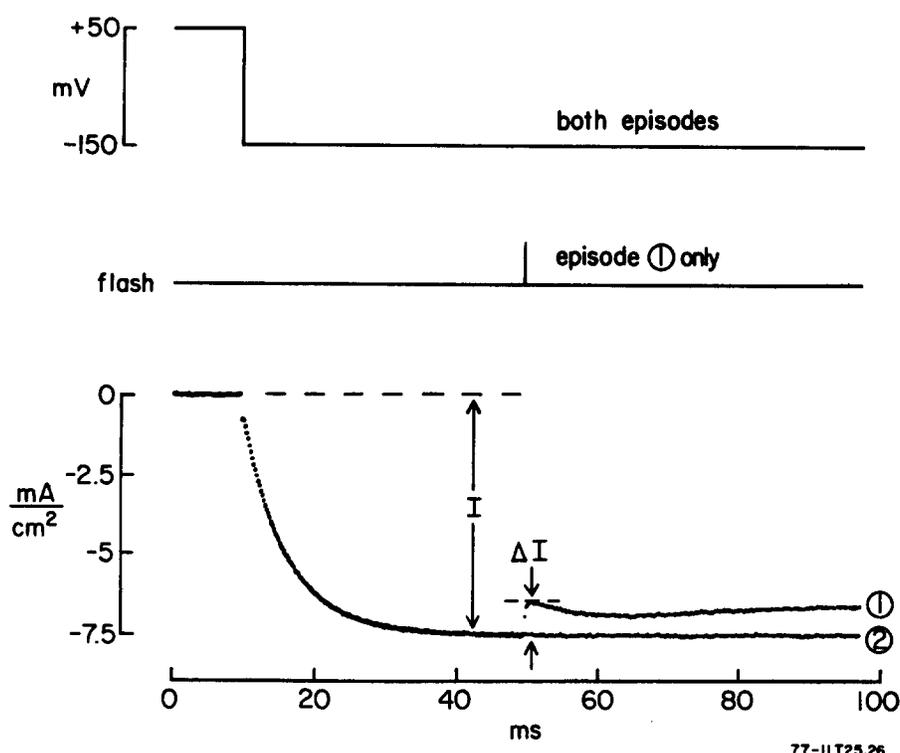


FIGURE 3. Currents induced in one electroplaque by low concentrations of *trans*-Bis-Q. Data are plotted on double-logarithmic coordinates, so that the slopes equal the Hill coefficient. Each point represents a separate application of agonist solution.

PHASES 2 THROUGH 4 Although this study concerns phase 1, there are several other interesting signals in Fig. 4. These have been analyzed in a previous study (Nass et al., 1978); an outline is given here for convenience. Before the episodes of Fig. 4, the preparation has been exposed to several laser

flashes. Therefore, the Bis-Q molecules are in a photostationary state ($\sim 66\%$ *trans*) and the laser flash of episode 1, while photoisomerizing individual Bis-Q molecules, has no net effect on this ratio. This explains why the agonist-induced current eventually returns to its pre-flash level after several seconds (episode 2).



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FIGURE 4. Voltage-jump and light-flash relaxations in the presence of Bis-Q (200 nM, *trans*-photostationary state). The trial comprises two voltage-clamp episodes, taken at an interval of 2 s. Lower traces show agonist-induced currents; leakage and capacitive currents have been subtracted. 100 ms after the start of the episode, the voltage is jumped from +51 to -150 mV. The agonist-induced current increases over the next 30 ms, reaching a steady state value labeled I . The voltage-jump relaxations superimpose for the two episodes. About halfway through the first episode, the flash occurs. The rapid decrease in agonist-induced current, labeled ΔI , is phase 1. The trial illustrated employs a relatively intense flash (0.69 flash^{-1}) for clarity. The data of Fig. 5 are taken with dimmer flashes. Temperature, 11°C . See text.

After phase 1, there is a conductance increase, termed phase 2. Phase 2 has the same time constant (~ 5 ms in the experiment of Fig. 4) as the voltage-jump relaxation that begins each episode. Phase 2 is the approach to equilibrium in response to the transient perturbation that produces phase 1 (Nass et

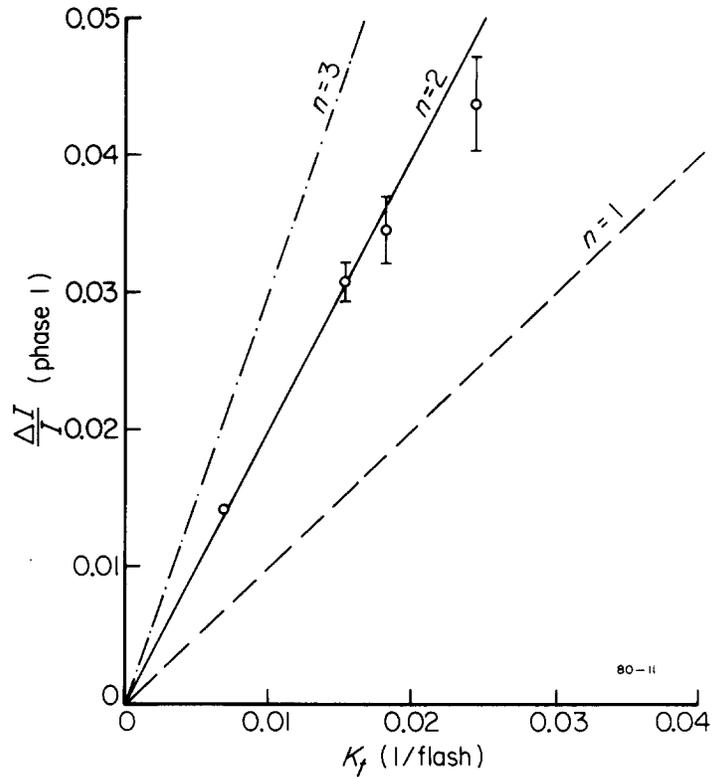


FIGURE 5. Amplitude of phase 1, $\Delta I/I$, vs. flash intensity, K_f , for one electroplaque at low flash intensities. See text.

TABLE II
PHOTON CROSS SECTION FOR PHASE 1

Cell	Temperature °C	n
78-11	22	1.47
79-21	23	2.28
79-22	23	2.21
79-23	23	2.25
79-24	24	2.20
79-51	23	2.18
79-53	23	2.29
79-61	10	1.85
79-62	9	2.13
80-11	9	1.74
Mean \pm SD		2.06 \pm 0.28

al., 1978). Over the next 40 ms, there is a slower decrease in current, termed phase 3. We have some evidence that this signal represents "open-channel blockade" produced by Bis-Q molecules that accumulate near receptors and are liberated by a flash (Nass et al., 1978; Lester et al., 1979, 1980). This

blockade disappears over the next few seconds; but the recovery, termed phase 4, is too slow to be recorded during episode 1.

Phase 1 Levels Off at High Flash Intensities

At flash intensities greater than $K_t = \sim 0.03 \text{ flash}^{-1}$, $\Delta I/I$ increases less than linearly with K_t , leveling off for $K_t \sim 0.2 \text{ flash}^{-1}$, corresponding to $K_c + K_t$

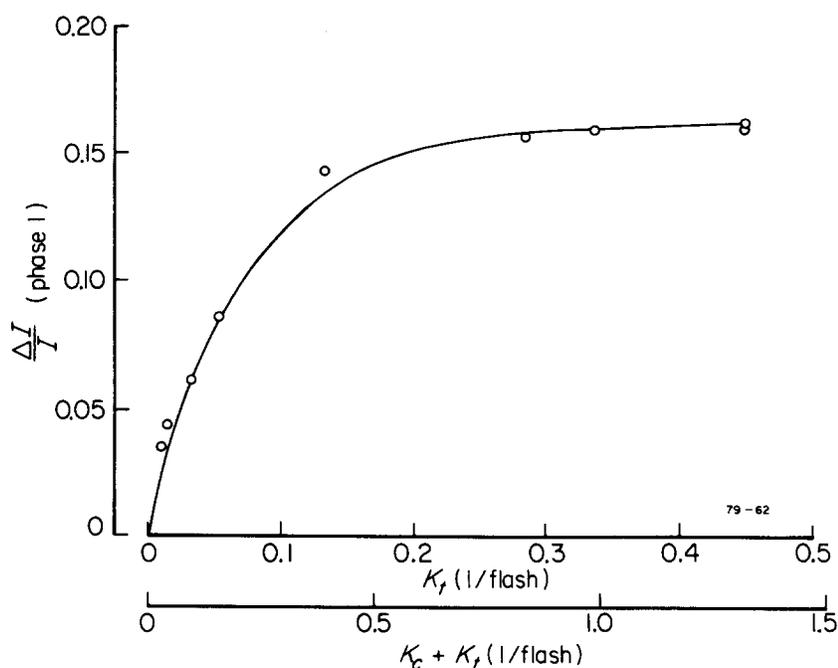


FIGURE 6. Amplitude of phase 1 vs. flash intensity for one electroplaque, showing leveling-off at high intensities. Temperature, 10°C.

TABLE III
ASYMPTOTIC VALUES FOR PHASE 1

Temperature °C	$\Delta I/I$ (mean \pm SEM; range)	Number of cells
10	0.141 \pm 0.018 (0.11-0.16)	7
23	0.209 \pm 0.016 (0.18-0.24)	9

Values given are asymptotic values for $\Delta I/I$, as in the experiment of Fig. 6.

$\sim 0.6 \text{ flash}^{-1}$. In the experiment of Fig. 6, for example, $\Delta I/I$ approaches an asymptotic value of 0.16. Table III gives the asymptotic values of phase 1 at two temperatures.

Two unsuccessful attempts were made to manipulate the asymptotic value.

For one cell tested at 22°C, phase 1 was measured both at the usual Bis-Q concentration of 200 nM and also in the presence of 1 μM D-tubocurarine with 1 μM Bis-Q. The value for $\Delta I/I$ was 0.21 in the former condition and 0.22 in the latter. Thus, the leveling off was not affected by curare, although we confirmed our previous observation that 1 μM curare eliminates phases 3 and 4 (Nass et al., 1978). For another cell at 14°C, we replaced the grating in the laser cavity with a totally reflecting mirror, thus producing unpolarized flashes. The asymptotic value for $\Delta I/I$ was 14%, no different from the value with the usual polarized flashes.

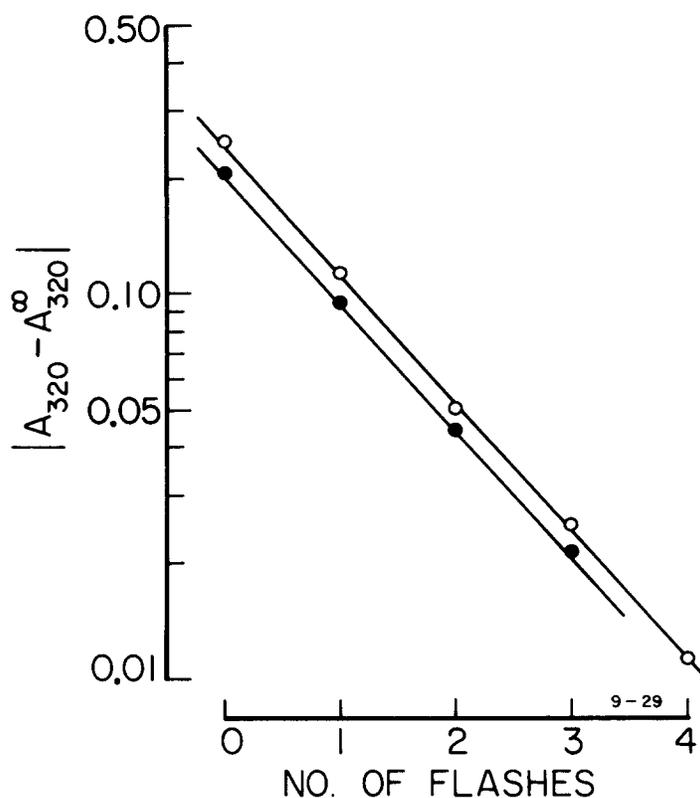


FIGURE 7. Semilogarithmic plots of the approach to photochemical equilibrium for QBr, beginning with the *trans*-isomer. Open circles, 10 μM solution. Closed circles, solubilized membrane fragments with tethered QBr.

Photoisomerization of Bound QBr

Spectral measurements were performed on the "tethered agonist" QBr (Bartels et al., 1971; Lester et al., 1980), covalently bound to acetylcholine receptors from *Torpedo* electric organ (see Methods). These spectra showed an amount of bound QBr between 0.45 and 0.55 times the amount of α -bungarotoxin binding sites on the membrane fragments, in good agreement with other

studies which have found half as many binding sites for tethered agonists as for α -toxins (Damle and Karlin, 1978; Moore and Raftery, 1979). Preincubation with α -bungarotoxin eliminated 75% of the QBr binding, which showed that most of the binding was to acetylcholine receptors. At a fivefold-higher QBr concentration (5 μ M), QBr and α -bungarotoxin bound at a ratio of 1.03, in agreement with the observations of Wolosin et al. (1980) that a second site for tethered agonists is revealed at higher concentrations. However, in this case less than half the binding was blocked by preincubation with α -bungarotoxin.

Laser flashes photoisomerized tethered QBr and unbound QBr to the same extent. In the experiment of Fig. 7, the apparent photoisomerization potency, $K_c + K_i$, was $1.30 \pm 0.03 \text{ flash}^{-1}$ for both tethered and free QBr.

DISCUSSION

The present results provide a straightforward confirmation of the concept that the open state of the acetylcholine receptor channel is much more likely to be associated with the continued presence of two bound agonist molecules than with a single such molecule. This conclusion is based on the demonstration that receptor channels close shortly after either of two bound agonist molecules are suddenly ($<1 \mu$ s) converted to a configuration that is no longer an agonist. The data are particularly satisfying because they pertain to the submillisecond time scale appropriate to the generation of the quantal event within the synaptic cleft (Wathey et al., 1979; Adams, 1980).

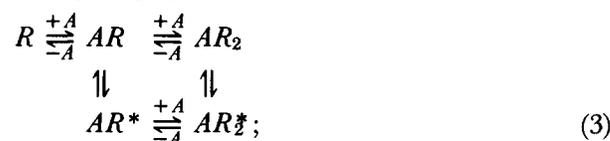
It may be asked whether these results rule out the possibilities (a) that channels can open, albeit with a low probability, with only one bound agonist (Dionne et al., 1978); and (b) that opening is even more likely with three bound agonists than with two (Dreyer et al., 1978). One approach to this question is simply to define the limits of our uncertainty for n . Several sources of experimental error are listed here with estimates of their contribution.

ERRORS DURING THE PHOTOCHEMICAL MEASUREMENTS (a) We do not precisely know the absorption spectra of the pure *cis* and *trans* Bis-Q (3%). (b) The slightly convergent laser beam shadowed part of the calibration cuvette (2%).

ERRORS DURING THE ELECTROPHYSIOLOGICAL MEASUREMENTS (a) The Ringer solution in pool A had a meniscus and acted as a lens (5%). (b) Flash intensity could be increased by reflections within the chamber (5%). (c) Flash intensity could be decreased because of absorption by connective tissue on the electroplaque surface (2%). (d) Conductances could be distorted by inaccurate compensation for series resistance or by inaccurate subtraction of leakage currents (5%). (e) The voltage-clamp circuit might be too slow to record accurately the peak of phase 1 (5%).

If one proceeds as usual to take the Pythagorean sum of these individual errors, one has a total uncertainty of $\sim 10\%$. Thus instead of the statement that the channel closes when either of two bound agonist molecules are photoisomerized, it would be more precise to say that "either of n " molecules are involved, where n is between 1.8 and 2.2. The 10% error limits leave room for a small contribution by monoliganded or triliganded receptors. This result

bears on the possibility raised by paragraph *c* in Theory. In the model,



the monoliganded channel can undergo the closed \rightarrow open transition, but under the conditions of our experiments, the data show that such an event would be followed, with at least 90% probability, by the binding of another agonist molecule.

We attempted to study monoliganded receptors more directly by producing phase 1 under conditions where such receptors would be expected to predominate, i.e., at small Bis-Q concentration or soon after a hyperpolarizing voltage jump as in the experiment of Fig. 4. However, the agonist-induced conductance is of course very small under such conditions; and because the experiments involved decrements of <5% in the signal, quantitative data were not available. We noticed no major changes in the saturation level of phase 1 under these circumstances.

These data may also be viewed as a test of the usual concept that the agonist molecules remain bound continuously while the channel remains open. Our photochemical manipulations require the presence of a photosensitive agonist molecule at the binding site; therefore, phase 1 would occur with diminished amplitude if this site is sometimes empty while the channel is open. If one accepts the assumption of exactly $n = 2$ stoichiometry, then this concept has been verified at the level of precision calculated above, i.e., ~10%. Thus, open channels have agonist bound at least 90% of the time.

The data do not bear directly on the finding that the two agonist molecules bind with different affinity constants (Sine and Taylor, 1980). In our experiments, one agonist molecule could indeed depart more readily than the other during spontaneous channel closures. Our theories about light-induced channel closures merely require (*a*) that *trans*-Bis-Q molecules at both binding sites be equally photolabile, and (*b*) that departure of either bound molecule of *trans*-Bis-Q closes the channel.

Photochemistry of Bound trans-Bis-Q

Our interpretation rests on the assumption that the spectral measurements of K_i apply to Bis-Q molecules bound to receptors, i.e., that *trans* \rightarrow *cis* photoisomerizations proceed with the same absorption spectrum and quantum yield for receptor-bound Bis-Q as for Bis-Q in aqueous solution. The spectral measurements on tethered QBr provide a partial test of this assumption. The remaining questions are (*a*) whether one can justifiably extrapolate from covalently bound QBr to reversibly bound Bis-Q; (*b*) whether measurements on *Torpedo* apply equally well to *Electrophorus* receptors; and (*c*) whether the Triton-solubilized receptors provide a close approximation to the receptors in the membrane. In view of the close pharmacological similarities between tethered QBr and reversible agonists (Lester et al., 1980), and between

acetylcholine receptors purified from different species (Conti-Tronconi et al., 1982), we do not think that important issues are raised by questions *a* and *b*. The third question is the most difficult. It does seem reasonable to assume that the agonist molecule's environment is determined mostly by a specific binding site on the receptor itself rather than by the membrane, but the question seems basically unresolvable by present techniques.

Saturation of Phase 1 at High Flash Intensities

It was surprising to find that phase 1 levels off, for high flash intensities, at values of 14% (10°C) or 21% (23°C). Values of >35% are observed in experiments where a flashlamp rather than a laser is used to generate the flashes (Nass et al., 1978). The laser flashes differ in three ways from those produced by a flashlamp: (*a*) the laser flashes are briefer (1 μ s vs. 500 μ s); (*b*) the laser flashes are monochromatic; (*c*) the laser flashes are plane polarized in most of our experiments. Point *c* was eliminated as a possible source of the leveling off in an experiment with unpolarized laser flashes. Of the remaining two possibilities, we have no definite basis for a choice but are inclined toward *a* because partially filtered flashes also gave large values for phase 1 in the previous flashlamp experiments (Nass et al., 1978). Thus, when phase 1 is produced by a flashlamp, the long flash duration might allow the conductance to approach a new equilibrium; on the other hand, the laser produces a truly instantaneous perturbation.

Even assuming that the saturation is linked to the brevity of the laser flashes, we have no satisfactory molecular explanation for the phenomenon. Very intense flashes produce a photostationary mixture of Bis-Q, containing 66% *trans*; and according to the theory presented, the ratio of active receptors just after the flash to those just before the flash ought to be $(0.66)^n$. Thus, $\Delta I/I$ ought to level off at $(1-0.66^n)$, which equals 34, 56, or 71% for $n = 1, 2,$ or 3, respectively. These are all larger than the observed value. Such a simple picture could, however, be complicated by the binding and diffusion of newly released Bis-Q molecules within the synaptic cleft. We feel that further study of this saturation might lead to new insights about such phenomena, but probably not about the basic mechanisms of agonist-receptor interaction.

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