PHOTOACTIVATION AND DISSOCIATION OF AGONIST MOLECULES AT THE NICOTINIC ACETYLCHOLINE RECEPTOR IN VOLTAGE-CLAMPED RAT MYOBALLS

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ABSTRACT The photochemical properties of the azobenzene derivative, Bis-Q, were exploited to carry out an agonist concentration jump followed by a molecular rearrangement of bound agonist molecules at acetylcholine (ACh) receptor channels of voltage-clamped rat myoballs. Myoballs were bathed in solutions containing low concentrations of *cis*-Bis-Q, the inactive isomer. Whole-cell current relaxations were studied following a light flash that produced a concentration jump of agonist, *trans*-Bis-Q, followed by a second flash that produced net *trans*→*cis* photoisomerizations of Bis-Q molecules. The concentration-jump relaxation provided a measure of the mean burst duration for ACh receptor channels occupied by *trans*-Bis-Q (7.7 ms, 22°C). The second current relaxation was a more rapid conductance decrease (phase 1, τ = 0.8 ms). Phase 1 may represent either the burst duration for receptors initially occupied by a single *cis*- and a single *trans*-Bis-Q molecule or that for unliganded receptors. Single-channel current recordings from excised outside-out membrane patches showed that single channels open following an agonist concentration jump comparable to that used in the whole-cell experiments; when many such records were averaged, a synthetic macroscopic relaxation was produced. Individual open channels closed faster following a flash that promoted *trans*→*cis* photoisomerizations of the bound ligand, thus confirming the whole-cell observations of phase 1.

INTRODUCTION

The photoisomerizable azobenzene derivative, Bis-Q, has made it possible to use light flashes to produce an agonist concentration jump at nicotinic acetylcholine (ACh) receptors (Lester and Chang, 1977). By bathing the preparation in solutions containing only the *cis* (inactive) isomer of Bis-Q, experiments can now be undertaken in which the resting conductance associated with a bath applied agonist has been reduced nearly to zero (Chabala et al., 1985*a*; Chabala and Lester, 1984, 1985). Because a brief flash of light is used to create agonist (*trans*-Bis-Q), both kinetic and steady-state information can be obtained as the occupancy of ACh receptors re-equilibrates following a true agonist concentration jump. Under those conditions, the concentration of agonist immediately adjacent to the receptors is known, and the usual problems posed by diffusion of agonist to the site of action are minimized.

Flashes can also be used to produce a direct perturbation of the agonist-receptor complex, as previous work has shown in isolated electroplaques from *Electrophorus electricus* (Nass et al., 1978; Sheridan and Lester, 1982). While still bound to the ACh receptor, a *trans*-Bis-Q molecule is photoisomerized to the *cis* isomer; newly created *cis*-Bis-Q then leaves the receptor site and the channel closes. The exact sequence of molecular events is uncertain, but in electroplaques the mean burst duration (see Colquhoun and Sakmann, 1981) is decreased by at least a factor of 100 compared with normal activation by *trans*-Bis-Q; receptor channels close within 100 μs. We have now used similar techniques to study both ACh channel activation and phase 1 in voltage-clamped rat myoballs (Horn and Brodwick, 1980). The details of both processes were further characterized by studying the behavior of currents through single ion channels recorded from excised patches of myoball membrane under similar conditions.

METHODS

The experimental methods are discussed in greater detail elsewhere (Chabala et al., 1985*a*; Lester et al., 1985). Briefly, mononucleated cultured rat myocytes (i.e., myoballs) were bathed in a solution containing *cis*-Bis-Q, the inactive isomer. The preparation of this isomer by high-performance liquid chromatography separation is described by Delcour et al. (1982) and by Nerbonne et al. (1983). Single-channel recordings in the presence of the *cis* isomer show very little activation of the ACh receptor channel (Chabala et al., 1982); the infrequent channel openings observed could have been caused by a small contamination by the *trans* isomer, or by openings of nonliganded receptor channels (Jackson, 1984). The experimental chamber containing the myoballs was mounted on the stage of an inverted microscope (Leitz Diavert; E. Leitz, Inc., Rockleigh, NJ). The chamber and temperature control circuit are described elsewhere (Chabala et al., 1985*b*). Two flash lamps were

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employed for the whole-cell experiments. Lamp No. 1 was mounted with its condenser at the camera port, near the intermediate image plane. The lamp housing had a Pyrex condenser that limited the output to wavelengths >350 nm; thus the output is termed "visible." Its light reached the preparation through the microscope objective (40×, NA 0.65). The details for measuring the photoisomerization rates, k_c for *cis*→*trans* and k_t for *trans*→*cis* photoisomerizations of the Bis-Q molecule are described by Chabala et al., (1985*a*) and by Sheridan and Lester (1982). Based on comparisons with a calibrated objective with an NA of 0.4 (see below), we estimated that the photoisomerization potency, $k_c + k_t$, was 4 flash^{-1} (see Chabala et al., 1985*a*, Nass et al., 1978, or Sheridan and Lester, 1982, for definition of inverse flash units), so that a single flash brought the isomeric composition to within 5% of the photostationary state (66% *trans*). Flash lamp No. 2 was mounted above the preparation, in the usual position of the microscope condenser. It was equipped with Ultraviolet (UV)-efficient quartz optics that passed wavelengths >250 nm and had an estimated photoisomerization potency of at least 1.4 flash^{-1} (Nargeot et al., 1982). The UV flux through the quartz optics resulted in a much greater fractional *trans*→*cis* photoisomerization potency, $k_t > 1.1$ flash^{-1} (Nargeot et al., 1982), because the molar extinction coefficients for the two isomers differ by a factor of ~20 in the UV region. As conditions were arranged so that the first flash brought the bath solution nearly to *trans* photoequilibrium, the second flash, therefore, produced predominantly *trans*→*cis* photoisomerizations. The single-channel experiments were carried out using an upright microscope (Leitz Dialux; E. Leitz, Inc.) in which the light flash was imaged onto the membrane patch through the microscope objective (32×, NA 0.40); the photoisomerization potency for those experiments was 1.7 flash^{-1}, and the k_t component, $1.7 \times k_t/(k_c + k_t)$, was ~0.58 flash^{-1}.

Standard voltage-clamp techniques were used to record whole-cell or single-channel currents (Hamill et al., 1981) following an agonist concentration jump or a molecular rearrangement of bound agonist molecules. For the whole-cell recordings, the chamber was perfused with a new *cis*-Bis-Q solution after two to three episodes and before recording from a new myoball. The experiments were carried out under yellow safelights (ML-2; Eastman Kodak Co., Rochester, NY). Flash-induced current relaxations were studied at low agonist concentrations; thus the time constants provide a measure of the mean channel burst duration (Colquhoun and Sakmann, 1981). An IBM personal computer interfaced with a Tecmar Lab Master board (Tecmar Inc., Cleveland, OH) was used to provide the voltage commands, control the light flashes, collect the current traces, and perform the data analysis (Kegel et al., 1985). The data were sampled at 100 or 200 μs/point. The external solution contained (in mM) 160 NaCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 5 glucose, and 5 HEPES-NaOH (pH = 7.3, ~290 mOsm/kg). The internal (pipette) solution contained (in mM) 140 CsCl, 10 EGTA-CsOH, 5 glucose, and 5 HEPES-CsOH (pH = 7.3, ~270 mOsm/kg). Tetrodotoxin (10^{-7} M) was added to the external solution to help suppress Na^+ currents. Outward currents at depolarized membrane potentials were not observed in the absence of a light flash; this suggests that there was good diffusional exchange between the myoball cytoplasm and the Cs^+ solution in the pipette. Series resistance compensation (1.5–2 times the electrode resistance) was used for cells in the whole-cell recording mode. The compensated passive charging current had a time constant of 0.2–0.5 ms, depending on the cell capacitance and series resistance of particular myoballs; this limited the fidelity of the voltage clamp to signals (in the worst case) with a time constant >0.5 ms.

RESULTS

Whole-Cell Recordings

Two whole-cell current relaxations are shown in Fig. 1. These traces show that there was no time-dependent ionic conductance until a flash was presented. Flash lamp No. 1 produced a localized concentration jump of agonist, *trans*-Bis-Q; ACh channels opened and the resulting current approached a steady state along an exponential time course (τ = 7.7 ms). A flash from lamp No. 2 was presented during the agonist-induced current, and two different effects were observed depending on the wavelengths of light presented. In either case, a biphasic relaxation was observed, and we interpret the rapid transient decrease in current as follows (Nass et al., 1978). The second flash caused a net photoisomerization of bound *trans*-Bis-Q to the *cis* isomer. The newly created bound *cis*-Bis-Q then left the receptor sites and channels closed, producing the fast decrease in conductance (phase 1, τ ~0.8 ms), which reflects the mean burst duration of receptors occupied by the normally inactive *cis* isomer (see below).

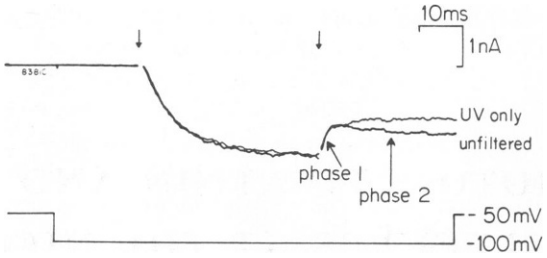

FIGURE 1 Whole-cell current relaxations in a voltage-clamped rat myoball bathed in a Ringer's solution containing 300 nM *cis*-Bis-Q. The membrane potential was stepped from a holding potential of −50 mV to a test potential of −100 mV, and a visible light flash was focused on the myoball through the microscope objective ~20 ms into the voltage step to create agonist (0 → 190 nM *trans*-Bis-Q). The time constant of each agonist-induced current relaxation was ~7.7 ms. A second flash (visible or UV only) was presented 40 ms later. The rapid component of the current relaxation following the second flash is called phase 1 (τ ~0.8 ms) and represents the photodissociation of bound *cis*-Bis-Q, as described in the text. The slower component is called phase 2 (τ ~8 ms) and represents the response of the ACh receptor channels to the second agonist concentration jump, as described in the text. Passive and capacitive currents have been subtracted. Both traces are the average of three separate responses. The solutions are given in the text, cell diameter = 30 μm, T = 22°C.

An additional effect of the second flash, however, was to change the local ratio of *cis*- to *trans*-Bis-Q in the bath. Following an unfiltered flash (λ >250 nm, the heavy trace in Fig. 1), additional *trans*-Bis-Q molecules from the bulk solution bind to the receptors, resulting in a conductance increase (phase 2) with a rate of activation similar to that following the first agonist concentration jump. The current did not completely recover to the value before the second flash, because it had a substantially greater UV component than the first flash (see Methods) and therefore produced a photoequilibrium state with a lower fraction of *trans* isomer. If, however, the agonist concentration jump was followed by a second flash filtered (UG 11; Schott Glass Technologies Inc., Duryea, PA) to transmit only UV light of 280–375 nm, a net local decrease in [*trans*-Bis-Q] was produced. A phase 1 decrease in current, with roughly the same amplitude as seen for the unfiltered flash, was again observed. As expected, the concentration-jump relaxation (phase 2) had approximately the same time constant (τ ~8

ms) as the initial agonist-induced current, although the relaxation was too small for precise measurements. Experiments with a filter (WG 295; Schott Glass Technologies Inc.) that transmits $\lambda > 295$ nm also showed an unaltered phase 1 as compared with the unfiltered flash. These experiments were carried out with similar results in several myoballs, and the flash effects were not seen with photostable agonists such as ACh.

Single-Channel Experiments

The single-channel counterpart of the whole-cell agonist concentration-jump experiment is shown in Fig. 2 *A*. The first 200 ms of each current trace (top sweeps in each set of four) show the typical low level of activity prior to the flash, while the remaining 600 ms of data (bottom three sweeps in each set of four) show the typical channel activity following the flash to create agonist. By averaging many such current traces (Fig. 2 *B*), it was possible to synthesize a trace that generally resembles the behavior of a macroscopic current relaxation following a similar agonist concentration jump. The decay of the averaged current record after about 150 ms is the expected behavior as the newly created agonist molecules diffuse away from the patch pipette tip.

Even with the comparatively narrow bandwidth (~1.5 kHz) used to record the single-channel currents in Fig. 2 *A*, it is clear that some open events are interrupted by brief closings; such gaps are more apparent at wider bandwidths (not shown). These units of openings inter-

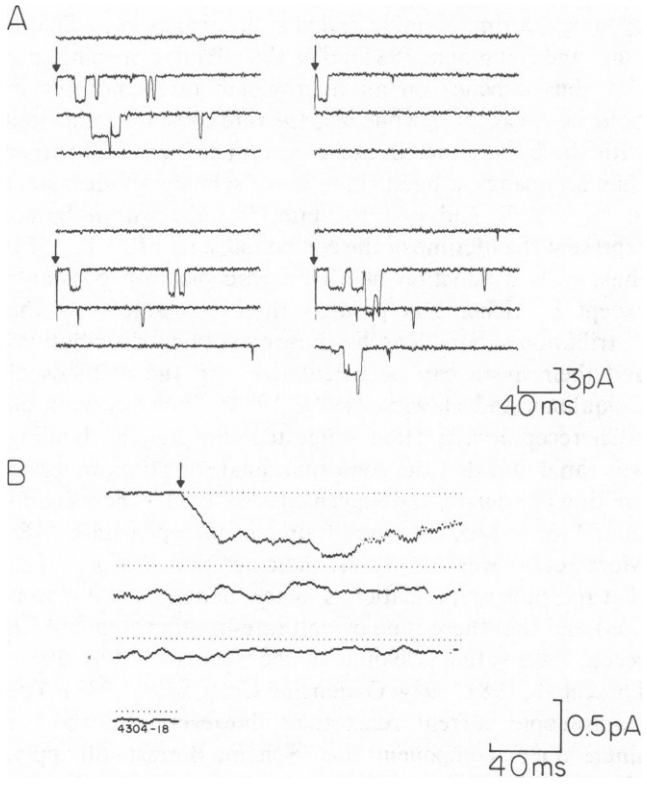

FIGURE 2 Response of single ACh channels to an agonist concentration jump. *A* shows four single-channel current records (each 800 ms long), before and after an agonist concentration jump (indicated by the arrow), in an excised outside-out membrane patch from a rat myoball bathed in 300 nM *cis*-Bis-Q. *B* shows the average of 250 single-channel current traces from the same myoball. The averaged current record rises to a steady-state, which is maintained until the newly created agonist molecules diffuse away into the bulk solution. Holding potential = −100 mV, T = 15°C.

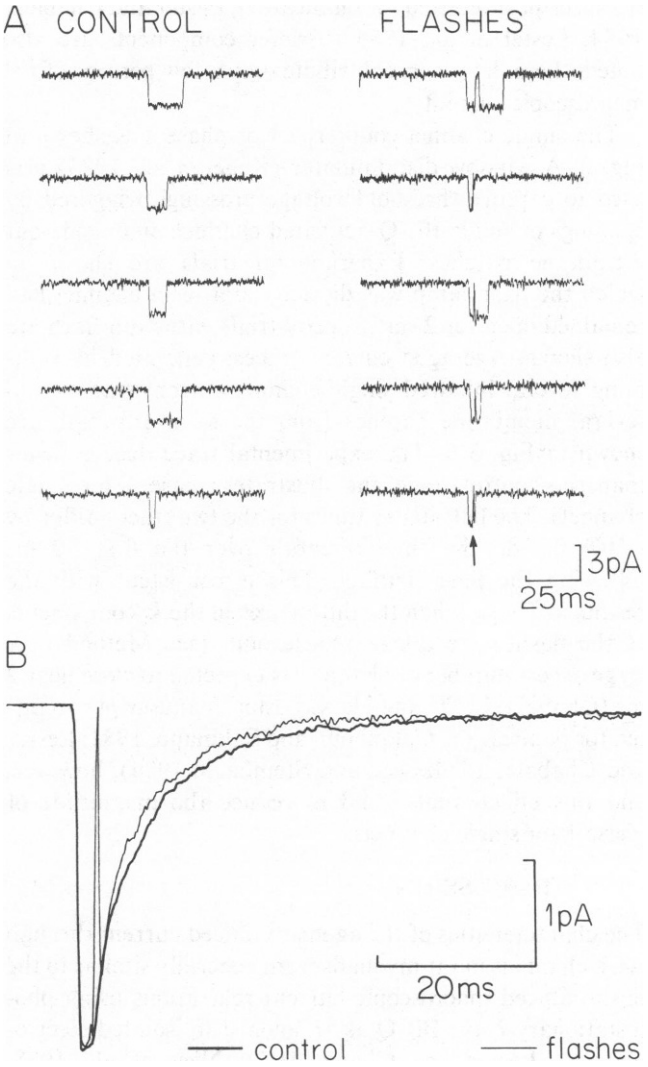

FIGURE 3 Response of single open ACh channels activated by *trans*-Bis-Q to light flashes. *A* shows typical single-channel current traces from an excised outside-out membrane patch from a rat myoball in the presence of 60 nM *trans*-Bis-Q in the bathing solution. During the experimental episodes, a flash was presented 2 ms after the channel had opened to photodissociate the bound agonist molecules. The flash lamp was allowed to recharge for at least 10 s between episodes. Control episodes, collected while the flash lamp recharged, also consisted of channels that were open for 2 ms or longer. Traces have been shifted in time so that the openings appear synchronous (halfway through the traces). Additional details are given in the text. *B* shows the average current trace generated by summing 317 flash trials and 572 control trials. The artificial flat top to these average current records was caused by the open-time criterion of 2 ms. Holding potential = −100 mV, T = 15°C.

rupted by a series of brief gaps are usually referred to as bursts (see Colquhoun and Sakmann, 1981), and the concept of burst duration has a precise meaning for specific models of receptor channel activation (see Colquhoun and Hawkes, 1981, 1982). When the distribution of burst durations from such records (as in Fig. 2 *A*) is analyzed, a slow component is found that generally corresponds to the time constants seen in the whole-cell relaxations under similar conditions (Chabala, L. D., and H. A. Lester, manuscript submitted for publication; Lester and Chabala, 1984; Lester et al., 1985). Briefer components are also noted; these, however, contribute only a few percent of the macroscopic current.

The single-channel counterpart of phase 1 is shown in Fig. 3. A window discriminator (Kegel et al., 1985) was used to capture threshold voltage crossings triggered by openings of single Bis-Q-activated channels in outside-out membrane patches. Experimental trials are shown in which the flash lamp was discharged after a channel had remained open for 2 ms. Control trials without a flash are also shown. Averaged current traces, generated by summing several hundred single-channel current traces from several membrane patches from the same myoball, are shown in Fig. 3 *B*. The experimental trace decays faster than the control trace and illustrates phase 1 for single channels. The half-decay times for the two traces differ by ~20%, as do the time integrals over the first 30 ms following the flash artifact. This is consistent with the results in Fig. 1, when the differences in the k_t components of the flashes are taken into account (see Methods). A large excess number of channels is expected to close near 2 ms (Chabala, L. D., and H. A. Lester, manuscript submitted for publication; Colquhoun and Sakmann, 1981; Lester and Chabala, 1984; Sine and Steinbach, 1984), however, and this effect might tend to reduce the magnitude of phase 1 for single channels.

DISCUSSION

The characteristics of the agonist-induced current through ACh channels in rat myoballs were generally similar to the flash-induced macroscopic current relaxations using photostationary *trans*-Bis-Q as an agonist in isolated electroplaques (Lester and Chang, 1977; Nass et al., 1978; Sheridan and Lester, 1982). The present experiments, however, were carried out by bathing myoballs in solutions containing only the *cis* isomer of Bis-Q (Nerbonne et al., 1983), and we have extended the observations to the level of single ion channels. The results show that macroscopic current relaxations following (*a*) an agonist concentration jump or (*b*) photodissociation of bound agonist molecules can also be understood in terms of the behavior of single ACh receptor channels under comparable conditions.

Interpretation of the Observations

Ongoing work in our laboratory indicates that at least 95% of the whole-cell conductance in rat myoballs can be accounted for by the sequential binding of two agonist (*trans*-Bis-Q) molecules (Chabala, et al., 1985*a*). Thus, a simplified molecular acivation scheme for ACh receptor channels can be written as:

$$2A + T \underset{k_{-1}}{\overset{2k_1}{\rightleftharpoons}} A + AT \underset{2k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_2T \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R, \qquad \text{(A)}$$

where A is the agonist, T represents a closed receptor, and R represents the open receptor; k_1 and k_2 are the rate constants for binding of agonist, while k_{-1} and k_{-2} are the rate constants for dissociation of the agonist; β is the rate constant for channel opening, and α is the rate constant for channel closing. Because the flash-induced current relaxations were characterized by a single time constant, the macroscopic channel gating mechanism can be simplified according to the following kinetic scheme:

$$S_1 \underset{\alpha'}{\overset{\beta'}{\rightleftharpoons}} S_2, \qquad \text{(B)}$$

where state S_1 represents the various closed states associated with agonist binding in Scheme A, and S_2 represents the compound burst state that arises from multiple channel openings during a single receptor occupancy (see Colquhoun and Sakmann, 1981); β' is the effective opening rate and thus depends on all microscopic rate constants in Scheme A except α, while α' is the rate constant associated with the burst state. Because current is measured rather than occupancy, a burst (in terms of scheme A) must start in state A_2R and exit to state T; gaps within bursts represent the lifetime of the compound state (T, AT, A_2T); thus, α' is a function of all microscopic rate constants except k_1. Scheme A predicts three components to the distribution of burst lengths; those conditional distributions and their mean can be calculated with the methods of Colquhoun and Hawkes (1981, 1982). Previous work on ACh receptor activation suggested that agonist binding was rapid and that the conformational transition was rate limiting (Anderson and Stevens, 1973). Under those conditions $1/\alpha' = 1/\alpha$, the true lifetime of the open state A_2R. More recent work, however, indicates that $\beta > k_{-2}$ (i.e., that receptor isomerization is faster than agonist dissociation) and that there is no overall rate-limiting step in ACh receptor activation (Colquhoun and Sakmann, 1981, 1983; Land et al., 1981, 1984; Ogden and Colquhoun, 1983). The macroscopic current relaxations, however, still show a single kinetic component; thus, Scheme B must still apply. Under those conditions, as calculations show using reasonable estimates of the microscopic rate constants for Scheme A, the major observable time constant of a whole-cell relaxation (i.e., the first flash in Fig. 1) should correspond to the mean burst duration rather than to the lifetime of the open state (see Colquhoun and Hawkes, 1982). Thus, at low agonist concentration, $\beta' \sim 0$ and

$$\tau \sim 1/\alpha', \qquad (1)$$

where τ represents the lifetime of the burst state for scheme B.

The second flash in Fig. 2 involves a molecular rearrangement of bound agonist molecules in the A_2R state. In previous studies on *Electrophorus* electroplaques, it was found that the channel closes if this rearrangement is a *trans* → *cis* photoisomerization (Nass et al., 1978). Photoisomerization of only one of the two bound agonist molecules is sufficient to close the channel (Sheridan and Lester, 1982). Because the *cis* isomer binds poorly to ACh receptors, it is not normally an agonist and presumably leaves the receptor soon after the photoisomerization. One can therefore write,

$$A_2R \xrightarrow{h\nu} AT + C, \qquad (C)$$

where A represents the *trans* isomer of Bis-Q and C represents the *cis* isomer. Present theories of receptor activation, however, shed little light on the exact sequence of events implied by the arrow. One interpretation is that phase 1 corresponds to the true lifetime of the burst state for receptors occupied by a single *cis*-Bis-Q and a single *trans*-Bis-Q molecule; thus,

$$A_2R \xrightarrow{h\nu} ACR \rightleftharpoons ACT \rightleftharpoons AT + C. \qquad (D)$$

The leftward arrows are short to denote that a weak agonist would have very small rates for activation and binding. Reopening would be unimportant, and a burst would be nearly equivalent to a single opening.

In another interpretation, the *cis*-Bis-Q molecule leaves first; thus,

$$A_2R \xrightarrow{h\nu} ACR \rightleftharpoons C + AR \rightleftharpoons AT, \qquad (E)$$

with the AR → AT transition rate-limiting, and phase 1 corresponds to the lifetime of the monoliganded open state AR. In this connection it is interesting to note that phase 1 has roughly the same time constant as (*a*) the brief component of channel opening due perhaps to monoliganded receptors (Colquhoun and Sakmann, 1981) or (*b*) the spontaneous openings (Jackson, 1984).

An interesting difference with previous work pertains to the kinetics of phase 1. In the present experiments with rat myoballs, phase 1 had a time constant of ~0.8 ms. In isolated electroplaques, phase 1 is about 10 times faster at similar voltages and temperatures (Nass et al., 1978). Because the molecular interpretation of phase 1 is uncertain, the reason for this difference is not clear. Our voltage clamp could not resolve a time constant ten times faster than phase 1 in rat myoballs, although the fidelity of the voltage clamp did not limit the response in these experiments. One possible pharmacological difference is that the receptors in electroplaques are mostly synaptic, while the receptors in developing myoballs are largely extrasynaptic; this difference in receptor type, possibly related to the 3–4-fold longer apparent mean lifetime for extrasynaptic receptor channels (Fischbach and Schuetze, 1980), could thus be the basis for a slower phase 1 in rat myoballs.

Trans-Bis-Q induces considerably larger conductances than does *cis*-Bis-Q in rat myoballs. For concentrations <1 μM, there are no complications introduced by open- or closed- channel block, and the difference in macroscopic conductance for the two isomers is at least a factor of 100 (Chabala, et al., 1985*a*). For a given concentration of *cis*-Bis-Q, the probability of an individual ACh receptor channel being open must, therefore, be at least 100 times smaller than for the same concentration of *trans*-Bis-Q. This difference is clearly not solely due to the measured 10-fold difference in burst durations. Thus, the effective opening rate must also be considerably smaller, although there are not yet enough data to account for the decreased rate in terms of particular molecular steps. One might, however, expect nonproductive binding to manifest itself as a block of closed channels (competitive or otherwise), but *cis*-Bis-Q is neither an antagonist nor an agonist of the ACh receptor in rat myoballs at concentrations <1 μM. This reinforces the assumptions of schemes *D* and *E* that the *cis* isomer has a considerably smaller forward binding rate at the receptor than does the *trans* isomer.

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