J. Physiol. (1986), **371**, *pp.* 407–433
With 12 text-figures
Printed in Great Britain

DOSE–RESPONSE OF ACETYLCHOLINE RECEPTOR CHANNELS OPENED BY A FLASH-ACTIVATED AGONIST IN VOLTAGE-CLAMPED RAT MYOBALLS

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(*Received 29 January 1985*)

SUMMARY

1. Whole-cell or single-channel currents through acetylcholine (ACh) receptor channels were studied in voltage-clamped rat myoballs or in excised membrane patches from myoballs. The recording pipette contained CsCl to suppress outward currents, and tetrodotoxin was used to help suppress Na^+ currents.

2. To minimize problems associated with bath applied agonists, myoballs were bathed in a solution containing the inactive (*cis*) isomer of the photo-isomerizable azobenzene derivative, Bis-Q. Calibrated light flashes of varying intensity were presented to produce concentration jumps of agonist, *trans*-Bis-Q. The resulting whole-cell current relaxations through ACh channels approach a steady state along an exponential time course, then decline as the newly created agonist diffuses away over the next few seconds.

3. The dose–response relationship was inferred from Hill (double-log) plots for myoballs bathed in 500 nM-*cis*-Bis-Q at three membrane potentials. At low agonist concentrations (< 300 nM-*trans*-Bis-Q), the slope of the Hill plot averaged 1·62 at −150 mV, 1·89 at −100 mV, and 2·05 at +80 mV. These results are consistent with an apparent agonist affinity constant that decreases with membrane depolarization and shifts the responses further down on the dose–response curve.

4. When the myoballs were bathed in higher concentrations of *cis*-Bis-Q (1·5–20 μM), the slope of the Hill plot was reduced at all membrane potentials, although it was still closer to two at positive potentials. This is expected from the known sigmoid shape of the dose–response relation. The shallow dependence of the Hill slope on agonist concentration suggests the presence of negative cooperativity in the over-all binding of agonist molecules.

5. Following treatment of the membrane with dithiothreitol to reduce disulphide groups, the Hill slope for the reversibly bound agonist, *trans*-Bis-Q, remained near two.

6. The kinetics of currents at hyperpolarized membrane potentials became complicated at higher agonist concentrations in a manner that was consistent with

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open-channel block by *trans*-Bis-Q; the currents showed a slow secondary increase in conductance.

7. Averaged single-channel recordings at higher agonist concentrations resemble macroscopic relaxations under comparable conditions. Furthermore, those recordings also suggested that open channels are blocked by *trans*-Bis-Q at concentrations $> 2\ \mu$M; the block depends strongly on membrane potential and increases with hyperpolarization. Currents at positive membrane potentials showed no evidence of open-channel block.

8. In the presence of 20 μM-*cis*-Bis-Q, the probability of a channel being open was constant from one episode to the next for up to several hundred flash-induced agonist concentration jumps (0 to 10·6μM-*trans*-Bis-Q).

9. Fractional activation of single ion channels at the highest agonist concentration tested (20 μM-*cis*-Bis-Q) was small, which agrees with the observation that whole-cell currents, under similar conditions, are still in the low concentration range of the dose–response curve.

INTRODUCTION

It has been clear since the classical work of Fatt & Katz (1951) on the properties of the frog motor end-plate that the primary response to activation of acetylcholine (ACh) receptors involves an increase in permeability to small ions. More recent work has shown that the primary response results from the opening of ion channels (Neher & Sakmann, 1976) whose selectivity filter is largely determined by an aqueous environment rather than by ion–protein interactions (Lewis & Stevens, 1983). The mechanism by which drug binding causes channel opening, however, remains only partially characterized (cf. Colquhoun, 1979). Initial observations on an S-shaped dose–effect relationship at the frog end-plate (Katz & Thesleff, 1957) have led to further studies concerning how many agonist molecules must bind to the receptor to open its ion channel (Karlin, 1967; Katz & Miledi, 1972) and to possible interpretations of non-integral estimates of that number (Colquhoun, 1973). Additional questions concern the role of agonist affinity for the receptor site (Ariëns, 1954) and the efficacy, or efficiency, of different agonists at opening the receptor channel (Stephenson, 1956). A number of authors have attempted to solve the first problem using various techniques to measure the conductance increase following agonist binding (reviewed in Colquhoun, 1979). Receptor desensitization (Katz & Thesleff, 1957), however, has been a major obstacle in most cases, and the observations have usually been restricted to hyperpolarized membrane potentials. The recent preparation of the inactive (*cis*) isomer of the photo-isomerizable azobenzene derivative, Bis-Q (Delcour, Cash, Erlanger & Hess, 1982; Nerbonne, Sheridan, Chabala & Lester, 1983), however, now permits the study of the steady-state dose–response relationship under conditions that combine the advantages of relaxation techniques (Adams, 1975*a*; Neher & Sakmann, 1975; Sheridan & Lester, 1975) with those of quantitative iontophoresis (Dionne, Steinbach & Stevens, 1978; Dreyer, Peper & Sterz, 1978).

In the present paper, whole-cell currents in voltage-clamped rat myoballs (Horn & Brodwick, 1980) were studied following a light flash that produced a concentration jump in agonist, *trans*-Bis-Q, from zero to a predetermined level. The slopes of

dose–response plots on double-logarithmic coordinates ('Hill' slopes) were determined for several agonist concentration domains across a wide range of membrane potentials (−150 mV to +80 mV). Three principal observations are made: the receptor is much more likely to be activated when two agonist molecules are bound to it than when a single one is bound; the Hill slope varies with membrane potential, suggesting that the over-all agonist affinity for the receptor is voltage dependent; and the Hill slope is decreased at all membrane potentials at higher agonist concentrations, in a manner that is consistent with negative co-operativity.

Preliminary results have previously appeared as an abstract presented to the Biophysical Society (Chabala, Gurney & Lester, 1984).

METHODS

Experimental preparation

Preliminary work indicated that the *trans* isomer of Bis-Q is an agonist in cultured chick and rat muscle and in the cloned cell line BC3H-1; the *cis* isomer, however, had little or no potency as an agonist (Chabala, Lester & Sheridan, 1982). The best preparation for combining whole-cell and single-channel work appeared to be rat muscle.

Primary cultures of thigh muscle myoblasts were prepared from neonatal Sprague–Dawley rats. Minced muscles were subjected to three 15 min incubations (at 37 °C) in Hank's Balanced Salt Solution (HBSS) containing 0·25% trypsin. The supernatant was discarded, and the muscle fragments were resuspended in Minimum Essential Medium (MEM) containing 1% L-glutamine, 1% penicillin–streptomycin, 10% fetal calf serum (FCS), and 1% chick embryo extract. The muscle fragments were dissociated by gentle trituration with a Pasteur pipette, and the suspension was filtered through a 25 μm nylon mesh. Fibroblasts were removed by pre-plating the cells in a large culture dish for 40 min at 37 °C. Myoblasts were counted and plated in 35 mm culture dishes containing 22 mm diameter glass cover-slips that were coated with rat-tail collagen. Thigh muscles from four to five pups were required for twenty culture dishes. The culture dishes were incubated at 37 °C in 5% CO_2. Multinucleated twitching myotubes formed from fused myoblasts within a few days. Many small spherical myocytes also remained, and these 'myoballs' were used for whole-cell or single-channel recordings. These myoballs are considerably smaller ($\sim$ 20 μm in diameter) than the multinucleated myoballs that have been used in other biophysical studies (cf. Horn & Brodwick, 1980). The cultures were maintained for several weeks by changing the maintenance medium (MEM, as above, but with 2·5% FCS) every two to three days.

Solutions

The bathing solution contained (in mM): 160, NaCl; 1·5, $MgCl_2$; 1·5, $CaCl_2$; 5, glucose, and 5, HEPES-NaOH (pH = 7·3, $\sim$290 mosmol/kg). Tetrodotoxin (TTX, 10^{-7} M) was added to help suppress Na^+ currents. TTX appears to have a low affinity for Na^+ channels in embryonic rat skeletal muscle or there is a population of TTX-resistant Na^+ channels; 10^{-7} M-TTX blocks only a fraction of the Na^+ current (L. D. Chabala, unpublished observations). To suppress outward currents, the recording/current-passing pipettes contained (in mM): 140, CsCl; 10, EGTA-CsOH; 5, glucose, and 5, HEPES-CsOH (pH = 7·3, $\sim$270 mosmol/kg). Without agonist, there were no outward currents at depolarized membrane potentials; this suggests that there was good diffusional exchange between the myoball cytoplasm and the pipette solution.

Experimental set-up

All experiments were carried out under yellow safelights (Kodak ML-2). Cover-slips with attached myoballs were transferred to a chamber, which was mounted on the stage of an upright microscope (Leitz Dialux, modified for fixed-stage operation with Reichert Nomarski optics). The chamber was housed in an insulated copper block cooled by two Peltier thermo-electric devices (Cambion No 3958-01) that transferred heat to circulating water. The temperature was usually maintained at 15±0·1 °C by a control circuit with feed-back from a linear thermistor (Yellow Springs

Instrument Co., No. 44202) mounted in the chamber. The chamber and control circuit are described in detail elsewhere (Chabala, Sheridan, Hodge, Power & Walsh, 1985). A xenon short-arc flashlamp, as illustrated in Fig. 1 *A*, was mounted in a shielded cage above the microscope so that flashes were imaged onto the myoballs through the objective (Leitz 32 ×, N.A. 0·4). For additional details see Lester, Chabala, Gurney & Sheridan (1986). Utraviolet light ($\lambda < 320$ nm) was absorbed by the flashlamp's Pyrex condenser, while longer wave-lengths ($\lambda > 800$ nm) were absorbed by an infra-red filter (Schott KG 1). Recharging of the flashlamp's capacitors was delayed ~250 ms after each flash to prevent pick-up by the current-to-voltage converter. In order to vary light intensity, and thus the magnitude of the agonist concentration jump, a series of neutral density filters were inserted into the light path. The diameter of the spot of light was controlled with a complete closure iris diaphragm (Rolyn Optics, Arcadia, CA), which was mounted near the flashlamp condenser at the intermediate image plane.

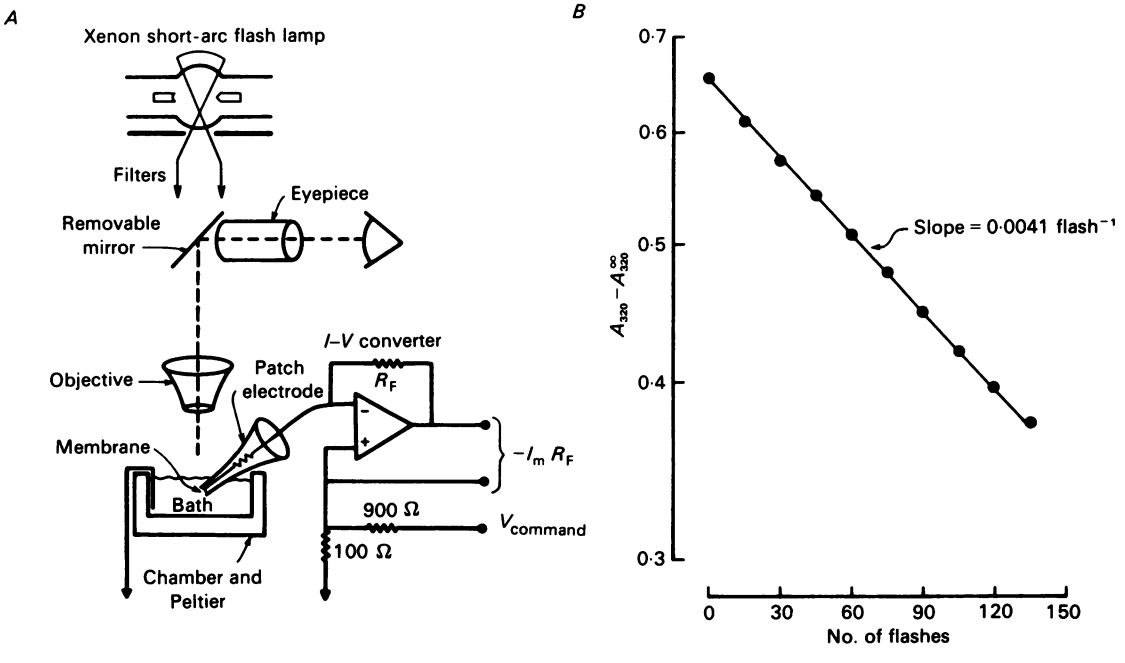

Fig. 1. *A*, schematic view of the apparatus for producing light flashes and recording whole-cell or single-channel currents. *B*, actinometric calibration series to determine the photo-isomerization potency for an agonist concentration jump. A cuvette, filled with 50 μM-*cis*-Bis-Q, was placed on the microscope stage in the path of a collimated beam of light and exposed to fifteen flashes. The absorbance, *A*, was measured at the absorption peak for *trans*-Bis-Q (320 nm), and the series was repeated several times. The absorbance was plotted *vs.* number of flashes, and a least-squares estimate of the slope provided a measure of the photo-isomerization potency. When the light was concentrated into a spot 50 μm in diameter through the microscope objective, the potency for a single flash was increased about 410-fold after correcting for light loss and vignetting effects.

Voltage-clamp techniques

Standard voltage-clamp techniques were used to record whole-cell or single-channel currents from ACh receptor channels (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). A Dagan 8900 Patch Clamp/Whole Cell Clamp was used to record whole-cell currents, while single-channel currents were recorded using a circuit similar to that described by Hamill *et al.* (1981). Voltage-clamp pipettes were pulled from soda-lime micro haematocrit tubes (Kimble 73811) and were coated with

a silicone elastomer (Sylgard 184) to reduce the capacitance to ground. Because the bathing solution contained only the *cis* isomer of Bis-Q, the background ionic conductance was close to zero; thus, the (usually low) leakage current in the whole-cell recording mode was subtracted electronically. Whole-cell currents were filtered at 3 kHz with an 8-pole Bessel low-pass filter. For single-channel experiments, the 'outside-out' patch configuration was used; these currents were usually filtered at 2 kHz. Current records were collected and stored during initial experiments with a Data General Nova 4 computer equipped with an ADAC analog interface; in later experiments, an IBM personal computer interfaced with a Tecmar Lab Master board (Kegel, Wolf, Sheridan & Lester, 1985) was used. Except where noted, the data were sampled at 200 μs/point.

Whole-cell frequency response and series resistance errors

The total cell capacitance, C_m, was estimated from the time integral of the charging current in response to a 20 mV hyperpolarization. These data were low-pass filtered at 6 kHz and digitized at a rate of 36 μs/point. The passive current response was described by a single exponential charging curve (cf. Horn & Brodwick, 1980) with a time constant $\tau \sim R_s C_m$, where R_s is the series resistance. For a 20 μm diameter myoball, C_m was typically about 40 pF and τ was approximately 400 μs; thus, R_s was roughly 10 MΩ, although the precise value varied considerably. The specific membrane capacitance was approximately 3 μF/cm^2, which suggests some membrane infolding (Horn & Brodwick, 1980). It was usually possible to compensate for about 50% of the series resistance without destabilizing the voltage clamp. The amount compensated was typically 1·5–2 times the electrode resistance (usually about 2–4 MΩ). The uncompensated series resistance could have been as large as 5–6 MΩ, which would introduce an error of about 5 mV/nA. This error could, in principle, distort both the kinetic and steady-state responses. The whole-cell currents, however, were generally < 1 nA; thus the voltage error was not more than a few mV in most instances. The compensated charging current had a time constant that could vary between 200–500 μs with different myoballs; hence, in the worst case, the fidelity of the voltage clamp was limited to signals with a time constant > 500 μs.

Calibration of light-flash potency

The photochemistry of Bis-Q has previously been described (see Lester & Nerbonne, 1982). High concentrations ($\sim$ 500 μM) of pure *cis*-Bis-Q were prepared and stored under liquid nitrogen (Delcour *et al.* 1982; Nerbonne *et al.* 1983). In a typical calibration experiment, absorption changes were monitored following a series of flashes that eventually produced a photostationary state (65% *trans*). The rate of approach to the photostationary state reflects the sum of the photo-isomerization potencies, k_c for *cis* to *trans* and k_t for *trans* to *cis* photo-isomerizations. These potencies are fully analogous to first-order unidirectional rate constants used in many biophysical models; the dimensions, however, are flash^{-1} rather than s^{-1}. They are defined so that an infinitesimally dim flash of potency $\delta k_{c,t}$ produces fractional *cis* to *trans* and *trans* to *cis* fluxes, respectively. Thus, a semilogarithmic plot of the approach to photochemical equilibrium has a 'rate' constant equal to $k_c + k_t$ (Sheridan & Lester, 1982). The calibration experiments integrate these relations in the natural way for real flashes. The steady-state fraction of the *trans* isomer is $k_c/(k_c + k_t)$, and the steady-state concentration of the *trans* isomer is $0{\cdot}65 \times [cis]_0$, where $[cis]_0$ is the initial concentration of the *cis* isomer.

Calibration experiments are not straightforward due to (a) the system's high numerical aperture and (b) the small test volume (25 nl) illuminated by the focused spot. Therefore, the strategy involved (1) increasing the beam intensity by opening the iris diaphragm to produce a 200 μm spot; (2) transforming the beam to a larger, nearly collimated one by removing the objective; (3) correcting for light loss in the objective; and (4) correcting for vignetting effects by the flashlamp's condenser. A custom made cuvette (see Sheridan & Lester, 1982) was filled with 50 μM-*cis*-Bis-Q, and the optical density was measured at the absorption peak for *trans*-Bis-Q (320 nm) and at the isosbestic point for *cis*- and *trans*-Bis-Q (266 nm). The cuvette was then placed in the experimental apparatus at the position of a myoball. The objective was replaced with a circular aperture that coincided with the entrance aperture of the objective (diameter = 5 mm) so that a collimated beam of light was projected through the cuvette. A series of fifteen flashes (15 s recharge time between flashes) were delivered; the cuvette was removed, and the optical densities were measured. This procedure was repeated 10–15 times. The cuvette was then exposed to steady illumination for about 5 min to produce the *trans* photostationary state, and the final optical density readings were taken.

The change in absorbance at 320 nm was plotted as a function of the number of flashes, as shown in Fig. 1*B*; the slope representing the photo-isomerization potency, $k_c + k_t$, was 0·0041 flash^{-1}. With the objective in place, a much more potent flash was created. If the objective was 100 % efficient, the photo-isomerization potency would have been increased by the ratio of the areas, to 2·56 flash^{-1}. Two independent methods were used to measure the efficiency of the objective. The beam from the objective was focused onto a bolometer (Scientech Laser Power Meter model 360001) or onto a photodiode (United Detector Technology, Inc. model PIN-10DB/541), and the energy produced by a single flash was measured with the iris diaphragm set to produce a 200 μm spot. For the measurements with the bolometer, a heat absorbing filter (Schott KG 1) was inserted into the light path. To protect the photodiode, neutral density filters were also used to block over 99·9 % of the light. The objective was then removed and replaced with a 5 mm circular aperture, and the measurements were repeated. Both sets of measurements indicated that the objective was about 52 % efficient.

Final corrections were made for vignetting effects. When the spot size was reduced by the iris diaphragm from 200 to 50 μm (a surface ratio of 16 : 1), the light intensity was reduced by only 12·6 : 1. Thus, we estimated that the photo-isomerization potency, $k_c + k_t$, for the standard 50 μm spot, was $2{\cdot}56 \times 0{\cdot}52 \times (16/12{\cdot}6) = 1{\cdot}69$ flash^{-1}. The concentration of *trans*-Bis-Q is given by

$$[trans] = 0{\cdot}65 \times [cis]_0 \times (1 - \exp\{-1{\cdot}69 \text{ flash}^{-1}\, nT\}), \tag{1}$$

where n is the number of flashes and T is the transmittance (i.e. $T = 10^{-D}$, where D is the optical density of the filter used to reduce the flash intensity). Previous work has shown that individual Bis-Q molecules isomerize within 1 μs after absorption of a photon (Sheridan & Lester, 1982), although the flash and its accompanying artifacts last for ~1 ms.

Approximations for the Hill coefficient

Hill coefficients (after Hill, 1910), originally used to characterize the oxygen-dissociation curve for haemoglobin, have also been used to estimate the number of agonist molecules that must bind to the ACh receptor to open its ion channel (see Colquhoun, 1973, 1979). The Hill coefficient, n_H, for the primary ionic conductance response can be defined as

$$n_H \frac{d \log \{[g(\lambda) - g(0)]/[g_{max} - g(\lambda)]\}}{d \log \lambda}, \tag{2}$$

where $g(\lambda)$ is the steady-state conductance at agonist concentration λ, and g_{max} is the maximum steady-state conductance at high agonist concentration. True Hill coefficients were not calculated because the true maximum steady-state conductance could not be measured due to the various complications at higher fractional activation (see Results). Most of the measurements were carried out at low agonist concentrations where $g(\lambda) \ll g_{max}$, in which case

$$n_H = \frac{d \log \{g(\lambda)\}}{d \log \lambda}, \tag{3}$$

since $g(0) \sim 0$ for myoballs bathed in solutions containing only the *cis* isomer of Bis-Q. Because large driving forces were used in the present paper, the difference between measuring steady-state current rather than steady-state conductance is unimportant; thus approximations to the Hill coefficient were provided by a least-squares estimate of the slope, on double-logarithmic coordinates, of steady-state current *versus* [*trans*-Bis-Q]. Eqn. (1) was used to calculate [*trans*-Bis-Q] following a flash. Nominally pure *cis*-Bis-Q solutions were periodically checked for contamination by the *trans* isomer of Bis-Q by measuring the ratio A_{320}/A_{266}. A small amount of contamination, generally less than 1 %, was usually present, and the dose–response curves were adjusted for this small effect. The change introduced by this correction increased the value of the Hill coefficient by 0·05–0·1.

RESULTS

Rationale for agonist concentration jumps

Rat myoballs were bathed in a solution containing a known concentration of *cis*-Bis-Q, which has a low affinity for the ACh receptor (Delcour *et al.* 1982; Nerbonne *et al.* 1983). The whole-cell steady-state conductance in the presence of 500 nM-*cis*-Bis-Q

was usually < 100 pS at −100 mV. Following a flash to produce agonist (0 to 270 nM-*trans*-Bis-Q), the steady-state conductance often increased to more than 10 nS indicating that the *trans* isomer is at least 100 times more potent as an agonist. Newly created agonist diffused away within a few seconds because the bath volume (~ 2 ml) was approximately five orders of magnitude larger than the volume occupied by the bolus of *trans*-Bis-Q. There are four advantages to this approach: the resting conductance is close to zero; agonist diffusion time to the receptor site is reduced; the concentration of agonist near the receptors is known, and receptor desensitization by the agonist is minimized.

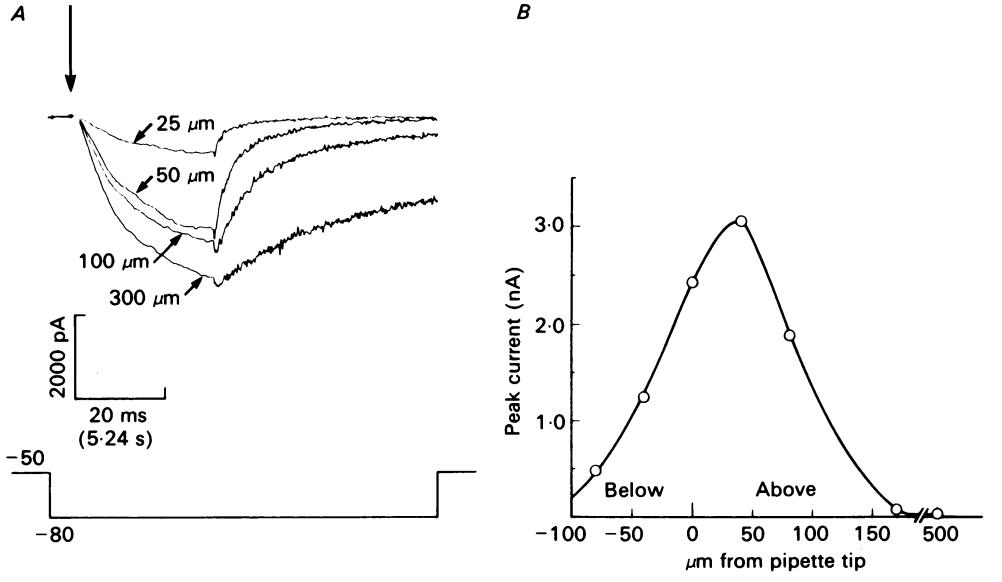

Fig. 2. Effects of spot diameter and plane of focus on agonist-induced currents. *A*, shows flash-induced currents for four spot diameters in the presence of 1·5 μM-*cis*-Bis-Q. The membrane potential was stepped from −50 mV to −80 mV for 13·62 s. A flash of variable diameter (indicated by the arrow) was imaged onto the myoball about 10 ms into the voltage step so that the effect of the agonist concentration jump and the corresponding half-time for agonist diffusion into the bulk solution could be measured. The first 256 points were sampled at 200 μs/point; the second 256 points were then sampled at 51·2 ms/point. *B*, conditions as in *A* except that the voltage step lasted 100 ms and the sample rate was 200 μs/point. A 50 μm spot was used, and the steady-state current was measured at several planes of focus. Temperature = 15 °C.

The influence of spot size and plane of focus on the response

The characteristics of the agonist concentration jump near the myoball depend on several factors including the diameter of the spot focused onto the myoball and the plane of focus of the spot. Whole-cell current relaxations produced by different spot sizes are shown in Fig. 2*A*. In this instance, the myoball was bathed in a solution containing 1·5 μM-*cis*-Bis-Q, and current relaxations for four different spot sizes are shown. For a spot diameter approximately equal to the cell diameter, the peak current was ~ 900 pA. When the spot size was approximately twice the cell diameter, the peak current increased substantially. Still larger spot sizes were associated with smaller increases in peak current. A split clock sampling rate was used for these

experiments; the faster sampling rate (200 μs/point) shows that the currents nearly reach a steady-state value within 50 ms; the slower sampling rate (51·2 ms/point) shows the time course for diffusion of agonist away from the myoball. As expected for diffusion in three dimensions, the removal of agonist did not follow a simple exponential time course. The half-times for removal varied markedly with the spot size, from less than 1 s at 25 μm to more than 10 s at 300 μm. These results suggested that the optimum spot size for activating currents in 20–25 μm diameter myoballs was approximately 50 μm; the corresponding half-time for agonist removal was about 2 s. Thus, the flash potency was calibrated for a 50 μm spot (see Methods).

The plane of focus also influenced the fraction of channels activated by the flash, as shown in Fig. 2*B*. In this instance, the plane of focus of the 50 μm diameter spot was varied and the steady-state current was measured. The largest steady-state current was obtained by focusing the spot approximately 50 μm above the surface of the myoball, presumably because no photo-isomerizations are produced by light within the glass slide.

The Hill coefficient at low agonist concentration

Flash-induced inward currents at -100 mV are shown in Fig. 3*A* for a myoball in the presence of 500 nM-*cis*-Bis-Q. Agonist concentration was varied by inserting neutral density filters into the light path (Fig. 1*A*), and two replications at each of three agonist concentrations are shown. The dose–response curve constructed from the steady-state currents using all the data for this myoball is shown in Fig. 3*B*. As expected at low agonist concentration when two agonist molecules must bind to open the receptor channel, the curve has a sigmoid start. The double-logarithmic plot of the same data is shown in Fig. 3*C*. In the low agonist concentration range, the Hill plots were usually quite linear and had very high correlation coefficients ($r \sim 0{\cdot}99$); there was no evidence that the response associated with the highest agonist concentration jump (i.e. unfiltered flashes) was saturating. A least-squares fit to the data of Fig. 3*C* yielded a Hill coefficient of 1·96; the average value of the Hill slope at -100 mV was $1{\cdot}89 \pm 0{\cdot}02$ (mean $\pm$ S.E. of mean, $n = 13$). In some (but not all) myoballs, a steady rundown of the agonist-induced current was observed during repeated flashes, amounting to about 40 % over the 30 min required for a complete experiment. Because the agonist concentration jumps used to estimate the Hill coefficients were carried out in known order and because the sequence was repeated 4–6 times during the period, rundown of the steady-state currents was not a significant problem.

A single time constant was observed in these current traces, which suggests two distinguishable states; thus the macroscopic currents can be characterized by a two-state scheme for receptor activation such as

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

where S_1 represents various closed states, including the agonist binding steps, and S_2 represents either the open state or the burst state (that could result from several closely spaced channel openings during a single receptor occupancy, cf. Colquhoun & Sakmann, 1981). For the present analysis, β', which increases with agonist

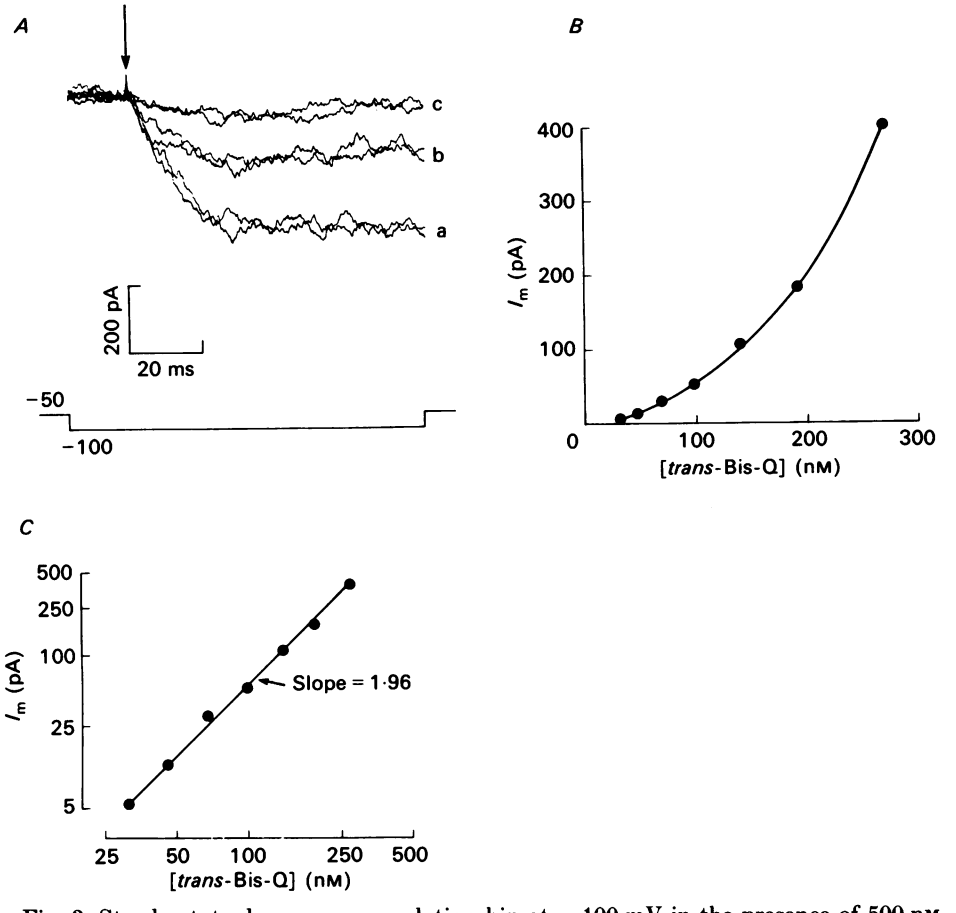

Fig. 3. Steady-state dose–response relationship at −100 mV in the presence of 500 nM-*cis*-Bis-Q. *A*, shows agonist-induced inward currents. The membrane potential was stepped from −50 mV to −100 mV for 100 ms. The flash produced a different agonist concentration jump depending on its intensity, and two currents at each of three agonist concentrations are shown (0 to 270 nM- (a), 0 to 190 nM- (b), and 0 to 100 nM- (c) *trans*-Bis-Q). Each current record is a difference trace in which a control episode (no flash) was subtracted from a light-flash episode. *B*, illustrates the dose–response relationship for the experiment in *A*. Four to six difference traces were averaged at each agonist concentration, and the steady-state response was determined by averaging the final 20 ms of each trace. *C*, shows the double-logarithmic plot of the data from *A*. The least-squares estimate of the Hill coefficient was 1·96 (r = 0·998). Temperature = 15 °C.

concentration, is the effective channel opening rate constant, while α' is the rate constant determined by the inverse of the mean channel or burst duration. The rate constants of the relaxations in Fig. 3*A* were similar ($1/\tau \sim 0{\cdot}07$ ms^{-1}), as would be expected at low agonist concentration; thus, $\beta' \ll \alpha'$ and, therefore, $1/\tau \sim \alpha'$.

Work is in progress regarding the voltage dependence of β' and α' in rat myoballs (Chabala & Lester, 1984, 1985). At low agonist concentration, the steady-state conductance is proportional to the ratio β'/α', and that ratio increases with hyperpolarization and decreases with depolarization. Thus, in order to assess the

influence of membrane potential on the Hill slope at low agonist concentration, experiments similar to those of Fig. 3*A* were carried out at more hyperpolarized and at more depolarized membrane potentials. The experiments designed to test for voltage dependence of the Hill coefficient were carried out at two membrane potentials in the same myoball (i.e. −150 mV and −100 mV or −100 mV and +80 mV), so a particular myoball generally served as its own control with regard to the voltage dependence of the Hill coefficient. At −150 mV, β'/α' increases, and the steady-state inward currents increase in magnitude; the Hill slope for myoballs bathed in 500 nM-*cis*-Bis-Q decreases to an average of about 1·62 ± 0·09 (mean ± S.E.

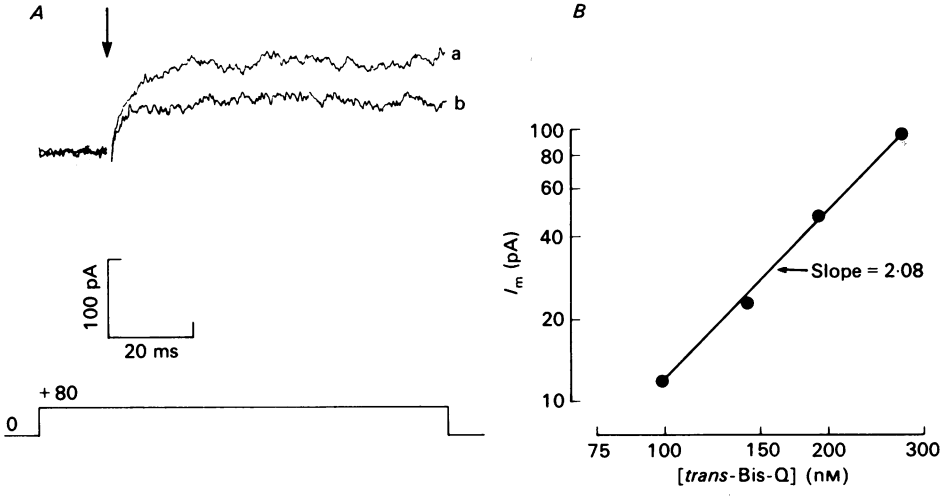

Fig. 4. Steady-state dose–response relationship at +80 mV. *A*, shows outward currents following agonist concentration jumps (0 to 270 nM- (a) and 0 to 190 nM- (b) *trans*-Bis-Q) in the presence of 500 nM-*cis*-Bis-Q. Each trace is the average of two difference episodes. The membrane potential was stepped from 0 mV to +80 mV. *B*, illustrates the double-logarithmic plot of all the data from *A*. Each data point is the average of four to six steady-state currents. The least-squares estimate of the Hill coefficient was 2·08 (r = 0·999). Temperature = 15 °C.

of mean, n = 4). In contrast to inward currents at large hyperpolarized potentials, outward agonist-induced currents at positive potentials are usually quite small because β'/α' decreases substantially with membrane depolarization (see also Horn & Brodwick, 1980). The use of Cs^+ in the recording pipette, however, blocks virtually all other outward currents, and flash-induced agonist concentration jumps produced easily measurable drug-activated outward currents, as shown in Fig. 4*A* at +80 mV. The Hill coefficient from the log-log plot (Fig. 4*B*) was 2·08. As in the experiment of Fig. 3*A*, the rate constants of the relaxations ($1/\tau \sim 0{\cdot}3$ ms^{-1}) did not change with agonist concentration. The average value of the Hill slope for myoballs bathed in 500 nM-*cis*-Bis-Q at +80 mV was 2·05 ± 0·02 (mean ± S.E. of mean, n = 5). Thus, the Hill slope depends on membrane potential at low agonist concentration; it is closest to 2 at positive membrane potential where receptor activation is smallest and decreases at hyperpolarized membrane potentials as receptor activation increases.

Concentration dependence of the Hill coefficient

The agonist concentration dependence of the Hill coefficient was explored by bathing myoballs in solutions containing higher concentrations of *cis*-Bis-Q. In principle, this should have shifted the underlying agonist-induced conductance increase further up on the dose–response curve; indeed, the currents were considerably larger at higher agonist concentrations (see Figs. 7 and 8). In general, the Hill coefficient was reduced at all membrane potentials at higher agonist concentrations (see Table 1), although it remained closer to two at positive potentials where the

TABLE 1. Hill coefficients

[*cis*-Bis-Q] (μM)	Membrane potential (mV)		
	−150	−100	+80
0·5	1·62 ± 0·09 (n = 4)	1·89 ± 0·02 (n = 13)	2·05 ± 0·02 (n = 5)
1·5	—	1·70 ± 0·01 (n = 3)	1·79 ± 0·04 (n = 3)
1·5*	—	1·73 ± 0·08 (n = 6)	1·89 ± 0·17 (n = 4)
5	—	1·68 ± 0·03 (n = 5)	1·81 ± 0·06 (n = 2)
20	—	1·45 ± 0·05 (n = 3)	1·61 ± 0·04 (n = 5)

* After DTT treatment.

relative receptor activation is smaller. In some instances, the Hill plots also showed evidence of departure from linearity and tended to become concave downward at the highest agonist concentrations. These effects are expected as the available binding sites become saturated with agonist, although, at hyperpolarized membrane potentials, channel block by the agonist (see below) could have similar effects. Rather surprisingly, however, the Hill coefficient showed only a shallow dependence on agonist concentration. As indicated in the Discussion, the roll off of the Hill coefficient at higher agonist concentration should have been much steeper (especially in the presence of 20 μM-*cis*-Bis-Q) if the second agonist molecule required to open the receptor channel binds more tightly than the first.

The effects of dithiothreitol

The Hill coefficient, using *trans*-Bis-Q as the agonist, was also estimated following treatment of the myoball with dithiothreitol (DTT) to reduce disulphide groups. Myoballs were exposed to 2 mM-DTT for 10 min before recording; to prevent spontaneous reoxidation during the experiment, both the bathing solution and the recording pipette contained 2 mM-DTT. Whole-cell currents were considerably smaller (~ 3–5-fold) following treatment with DTT, thus the concentration of *cis*-Bis-Q in the bath was increased to 1·5 μM; α', however, still dominated the current relaxations at this concentration. In addition to the several-fold diminution of the currents, the kinetics were faster ($1/\tau \sim 0{\cdot}2$ ms^{-1} at −100 mV) following treatment with DTT, as shown in Fig. 5*A*. These currents were part of the dose–response series plotted in Fig. 5*B*, which had a Hill coefficient of 1·73. Similar results were obtained at +80 mV (Table 1).

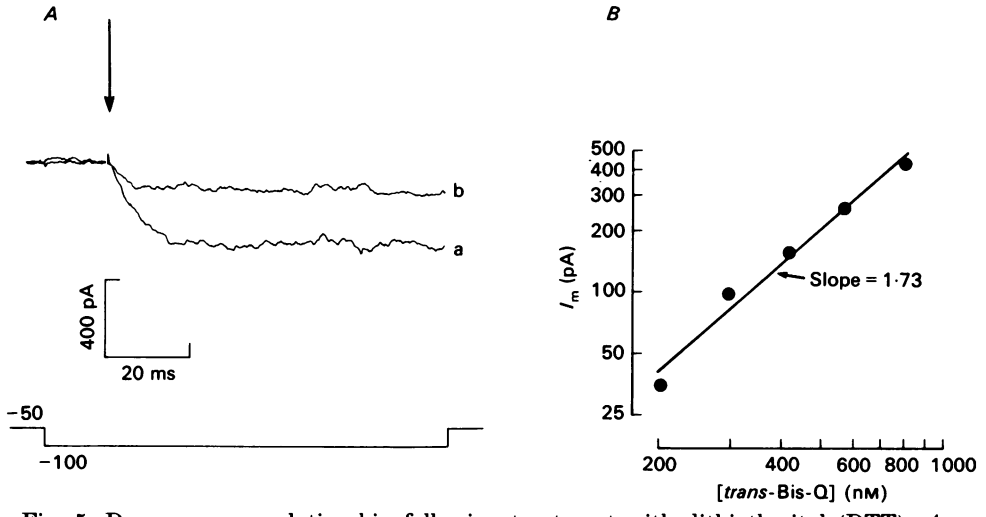

Fig. 5. Dose–response relationship following treatment with dithiothreitol (DTT). *A*, typical difference current traces following two agonist concentration jumps (0 to 270 nM- (a) and 0 to 100 mM- (b) *trans*-Bis-Q) in the presence of 1·5 μM-*cis*-Bis-Q during a voltage step from −50 mV to −100 mV. *B*, shows the double-logarithmic plot for all agonist concentration jumps. Steady-state responses were computed after averaging three to four difference traces. The least-squares estimate of the Hill coefficient was 1·73 (r = 0·988). Temperature = 15 °C.

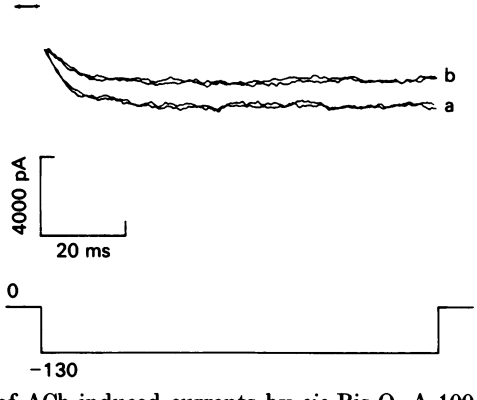

Fig. 6. Antagonism of ACh-induced currents by *cis*-Bis-Q. A 100 ms voltage step from 0 mV to −130 mV in the presence of 1·5 μM-ACh produced inward currents, and the two superimposed traces were recorded at an interval of 2 s (a). A solution containing 1·5 μM-ACh + 5 μM-*cis*-Bis-Q was then perfused into the chamber, and the voltage step was repeated. The *cis* isomer of Bis-Q reduced the ACh-induced current by about 30% (b). Temperature = 22 °C.

Channel activation at higher agonist concentration

As a prelude to working with higher agonist concentrations, we investigated the effects of *cis*-Bis-Q on agonist-induced currents. Based on the results with the closely related competitive antagonist 2BQ (Krouse, Lester, Wassermann & Erlanger, 1985), the *cis* isomer of Bis-Q might be expected to antagonize agonist-induced currents at higher concentrations. As shown in Fig. 6, 5 μM-*cis*-Bis-Q suppressed about 30% of

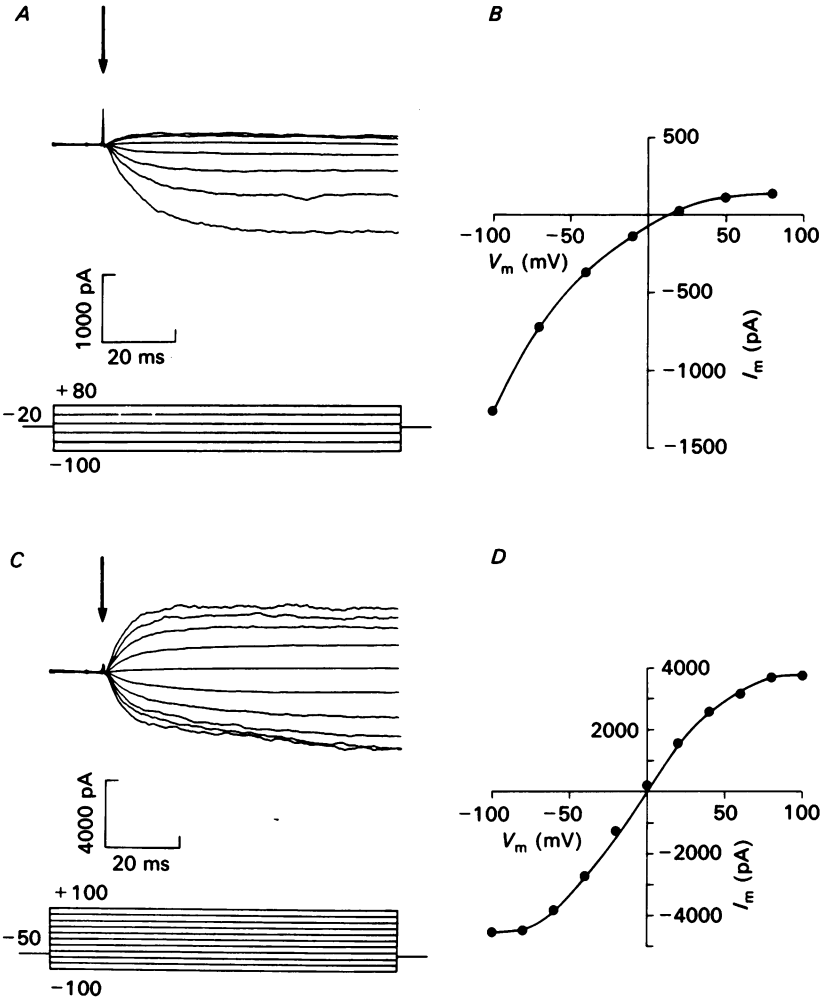

Fig. 7. Current–voltage (I–V) relationship. A, shows flash-induced currents at various membrane potentials (from a holding potential of −20 mV) in the presence of 1·5 μM-*cis*-Bis-Q. The agonist concentration jump was from 0 to 810 nM-*trans*-Bis-Q. B, shows the steady-state I–V relationship from A. C, shows flash-induced currents (holding potential of −50 mV) in the presence of a high (30 μM) concentration of *cis*-Bis-Q. D, currents in C were averaged over the last 10 ms and plotted against membrane potential to generate the isochronal I–V relationship, which shows that inward currents are attenuated. Temperature = 15 °C.

the steady-state current induced by ACh. The kinetics of averaged ACh-induced relaxations were similar before ($1/\tau = 0{\cdot}16 \pm 0{\cdot}004$ ms^{-1}, $n = 6$) and after ($1/\tau = 0{\cdot}15 \pm 0{\cdot}004$ ms^{-1}, $n = 6$) *cis*-Bis-Q was perfused into the chamber. We have not studied this antagonism in detail; it is possible, however, that *cis*-Bis-Q competes for the agonist binding site, on a time scale either much faster or much slower than our measurements (cf. Colquhoun, Dreyer & Sheridan, 1979).

Flash-induced currents recorded at low agonist concentration and various membrane potentials are shown in Fig. 7 A. These currents all approached the steady state with

a single exponential rate constant that varied from $1/\tau \sim 0{\cdot}08$ ms^{-1} at -100 mV to $1/\tau \sim 0{\cdot}28$ ms^{-1} at $+80$ mV. The usual steady-state current–voltage (I–V) relationship is shown in Fig. 7*B*. At higher agonist concentration, however, the kinetics of the currents became more complicated, as shown in Fig. 7*C*. Outward current relaxations at all agonist concentrations tested were well fit by a single exponential component and reached a steady state within a few milliseconds. Inward currents at higher agonist concentrations, however, tended to relax with two components; the slower phase did not reach a steady state in the time scale examined. The isochronal I–V relationship is shown in Fig. 7*D*. A voltage-dependent reduction in inward current is apparent, an effect that is consistent with open-channel block by the agonist.

Channel block was less apparent with weaker flashes. Outward currents recorded at $+70$ mV are shown for a moderate (Fig. 8*A*) and a small (Fig. 8*B*) agonist concentration jump in the same myoball. Both agonist-induced outward currents are reasonably well described by a single exponential component ($1/\tau \sim 0{\cdot}48$ ms^{-1} in Fig. 8*A* and $1/\tau \sim 0{\cdot}29$ ms^{-1} in Fig. 8*B*). The inward current at -100 mV for the moderate agonist concentration jump (Fig. 8*A*) showed two components. When the light intensity was reduced by a factor of 8 by inserting a neutral density filter into the light path (as in Fig. 8*B*), the inward currents were characterized by a single exponential component ($1/\tau \sim 0{\cdot}07$ ms^{-1}). Because neutral-density filters allow the experimenter to manipulate the size of the concentration jump of *trans*-Bis-Q while the total Bis-Q concentration remains constant, the series shows that the complicated kinetics (as in Fig. 8*A*) are largely caused by the *trans* isomer.

It is shown more clearly in Fig. 9 that $1/\tau$ increased with agonist concentration. This myoball was bathed in a higher concentration of *cis*-Bis-Q (30 μM) and exposed to flashes of different intensity. Currents produced by four agonist concentration jumps, at two membrane potentials, are shown in Fig. 9*A*, and the rate constants estimated from the relaxations are plotted against the complete agonist concentration series in Fig 9*B*. The extrapolated zero-concentration estimates of $1/\tau$ generally agree with the rate constants measured at low agonist concentration (as in Figs. 3 and 4). The increase in $1/\tau$ with agonist concentration can be partially attributed to an increase in β', although open-channel block by the agonist would also be expected to accelerate the rates for inward currents.

Single-channel recordings support open-channel block by the agonist

Single-channel recordings under the conditions of the present experiments show virtually no short latency responses during a 100 ms voltage step when visible light to the preparation is blocked with a filter; thus channel activity following an agonist concentration jump is almost exclusively due to the binding of newly created agonist. The channel openings are clearly time-locked to the light flash. At lower agonist concentration the major component of the distribution of burst lengths has a mean of 10–14 ms (Chabala, Gurney & Lester, 1985; Lester *et al.* 1986). Fig. 10 presents an experiment at high agonist concentrations. Inward current traces at -100 mV from an outside-out membrane patch are shown following an agonist concentration jump from 0 to 10·6 μM-*trans*-Bis-Q. It was clear that the fraction of brief openings increased. There were virtually no single openings longer than ~ 7 ms; the average duration for single openings was $1{\cdot}90 \pm 0{\cdot}10$ ms (2370 openings). These single openings,

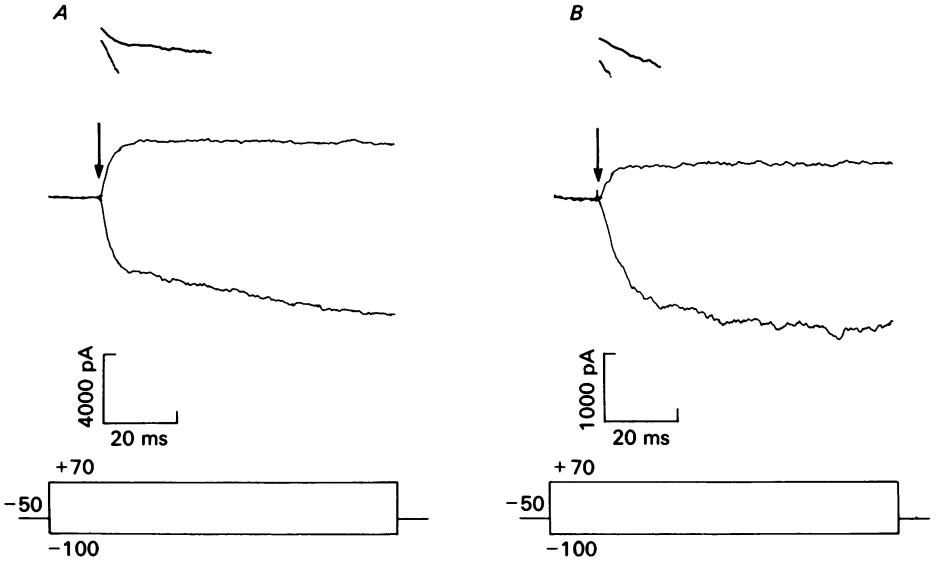

Fig. 8. Removal of apparent channel block by weaker flashes. *A*, difference currents at two membrane potentials (−100 mV and +70 mV, holding potential = −50 mV) in the presence of 30 μM-*cis*-Bis-Q following an agonist concentration jump from 0 to 15·9 μM-*trans*-Bis-Q. The inward current at −100 mV shows apparent channel block, as in Fig. 7*C*. *B*, difference currents in the same myoball but following a lower agonist concentration jump (0 to 2·5 μM-*trans*-Bis-Q). The reduced flash intensity reduced the apparent channel block of the inward current at −100 mV. Temperature = 15 °C.

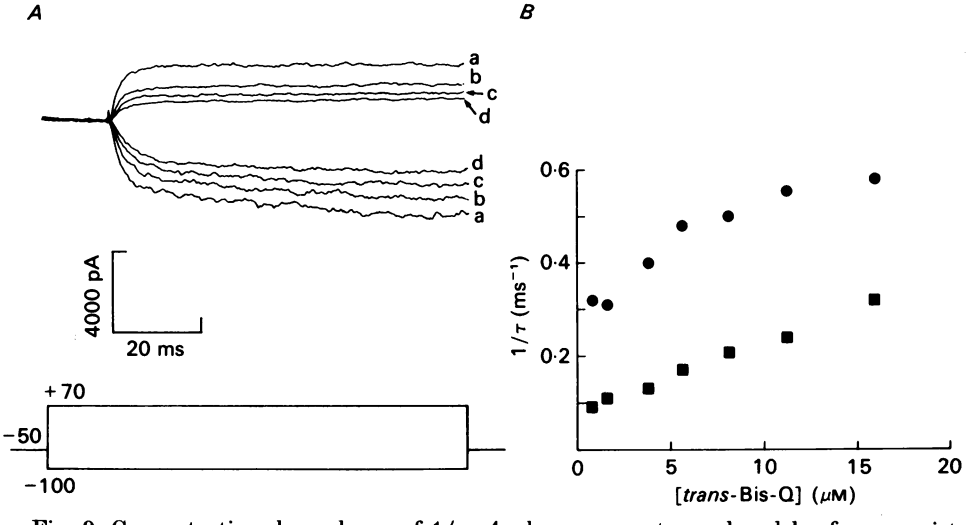

Fig. 9. Concentration dependence of $1/\tau$. *A*, shows currents produced by four agonist concentration jumps (0 to 15·9 μM- (a), 0 to 11·2 μM- (b), 0 to 8·1 μM- (c), and 0 to 5·6 μM- (d) *trans*-Bis-Q) at +70 mV and −100 mV from a holding potential of −50 mV in the presence of 30 μM-*cis*-Bis-Q. *B*, shows a plot of $1/\tau$ *vs.* [*trans*-Bis-Q] from the experiment in *A*. Inward currents did not reach a steady state, thus, $1/\tau$ was estimated by fitting the approach to a sloping steady-state line. The filled circles are the data at +70 mV, while the filled squares are the data at −100 mV. Each point is the mean of two observations. Temperature = 15 °C.

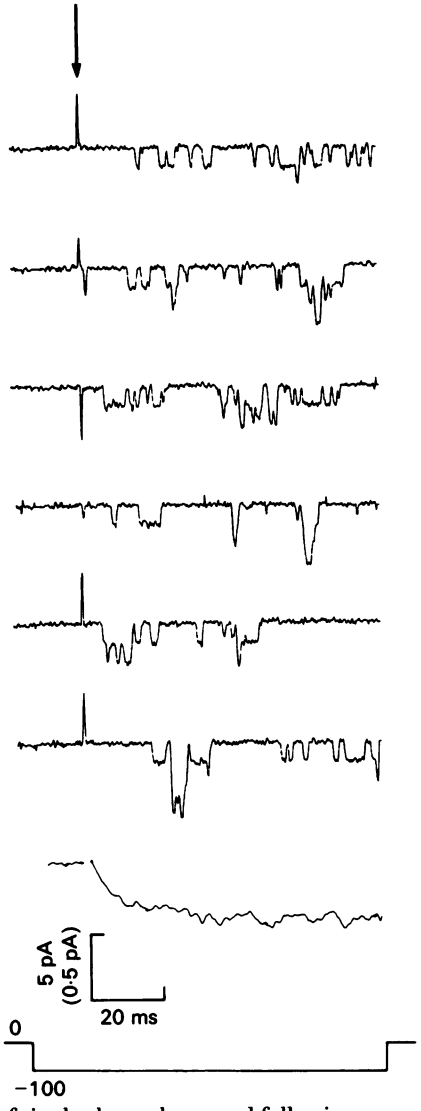

Fig. 10. Characteristics of single channels opened following an agonist concentration jump from 0 to 10·6 μM-*trans*-Bis-Q in an outside-out membrane patch. The membrane potential was stepped from 0 mV to −100 mV, and current records were collected in blocks of sixteen episodes/trial. Visible light was blocked for the first episode, and this control episode was subtracted, to remove leakage current and the capacity transient, from each experimental episode. Six difference traces are shown. The average of 237 difference traces from the same patch is shown below the single-channel current records. Temperature = 15 °C.

however, are not necessarily synonymous with a single sojourn in the burst state (cf. Colquhoun & Hawkes, 1981). The attenuation of open time increased strongly with membrane hyperpolarization. At −190 mV under the conditions of Fig. 10, for instance, most measurable open durations were < 2 ms. We interpret these data as evidence for open-channel block by the agonist. At large hyperpolarized membrane potentials, either isomer, at higher concentrations, can cause open-channel block,

although the *trans* isomer is more effective (L. D. Chabala & H. A. Lester, unpublished observations). The average of 237 single-channel current traces from the same membrane patch is also shown in Fig. 10. Two components, typical of a macroscopic current relaxation at this concentration, are evident; the slow component is more pronounced at higher agonist concentrations. At lower agonist concentrations (as in Chabala *et al.* 1985; see also Lester *et al.* 1986) or at positive membrane potentials (not shown), the averaged record over the first 100–150 ms usually shows a single exponential component.

A model for channel block by Bis-Q

Due to the voltage sensitivity of the dose–response relation, fractional receptor activation is highest under the conditions of Fig. 7*C* at hyperpolarized membrane potentials. This is of course the region where complicating effects of the agonist, such as open-channel block, would affect the results most. The simple three-state scheme proposed by Adams (1976) to explain open-channel block by the agonist can be represented as

$$S_1 \underset{\alpha'}{\overset{\beta'}{\rightleftharpoons}} S_2 \underset{k_{32}}{\overset{k'_{23}}{\rightleftharpoons}} S_3, \tag{B}$$

where S_1 is the closed state, S_2 is the burst state, S_3 is the open-blocked state, k'_{23} is the open-channel blocking rate, and k_{32} is the rate of unblocking. In this model, β', the effective opening rate, increases as the square of agonist concentration, while k'_{23} increases linearly with agonist concentration. The concentration dependence of these terms is given by

$$\beta' = [\textit{trans}\text{-Bis-Q}]^2 k_{12}/(K + [\textit{trans}\text{-Bis-Q}]), \tag{4}$$

$$k'_{23} = [\textit{trans}\text{-Bis-Q}]\, k_{23}, \tag{5}$$

where k_{12} and k_{23} are the bimolecular binding rates (with units of $\mu M^{-1}\ ms^{-1}$), and K is the apparent dissociation constant. In order to simplify the model, the agonist concentration dependence of β' was assumed to be that given by scheme C below, in which the binding of the second agonist molecule directly opens the receptor channel (cf. Sheridan & Lester, 1977). According to the classical sequential model, equilibrium conductances are not affected at low agonist concentration but are progressively reduced as the agonist concentration is increased (see below), especially at larger negative membrane potentials.

The lifetime of the burst state, τ_2, for scheme B is given by

$$\tau_2 = \frac{1}{\alpha' + k'_{23}}. \tag{6}$$

Thus, the mean burst duration should decrease with agonist concentration due to open-channel block by the agonist; by contrast, in the absence of open-channel block, the mean burst duration (for a more detailed molecular model) would be expected to increase with agonist concentration (cf. Colquhoun & Hawkes, 1981). In addition, previous work (Neher & Steinbach, 1978) suggests that k_{23} should depend strongly on membrane potential, increasing with hyperpolarization; thus open-channel block should increase with hyperpolarization and decrease with depolarization, observations

that were borne out in the present work. We note, as well, that there is an expected iontophoretic (or current-dependent) effect, which would have similar channel-blocking consequences. Bis-Q bears two positive charges; thus, inward current flow would tend to carry Bis-Q into the channel where it could block ion flow, while outward current would tend to prevent Bis-Q entry into the channel. Accordingly, our data were fit well with a blocking rate constant k_{23} of 0·025 μM^{-1} ms^{-1} at −100 mV and zero at +70 mV.

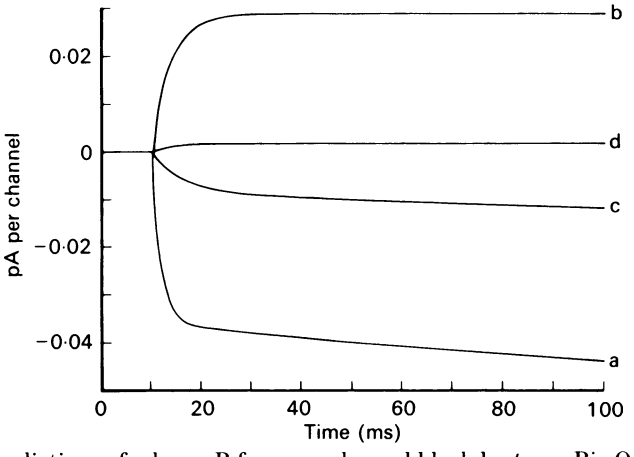

Fig. 11. Predictions of scheme B for open-channel block by *trans*-Bis-Q at two membrane potentials, −100 mV and +70 mV. In order to match the conditions of Fig. 8, the agonist concentration jump for traces a and b is 0 to 18 μM-*trans*-Bis-Q. For traces c and d, the agonist concentration jump is 0 to 3 μM-*trans*-Bis-Q. Channel opening is described by $k_{12} = 5 \times 10^{-4}$ μM^{-1} ms^{-1} and $\alpha' = 0{\cdot}07$ ms^{-1} (at −100 mV) or $k_{12} = 1{\cdot}5 \times 10^{-4}$ μM^{-1} ms^{-1} and $\alpha' = 0{\cdot}25$ ms^{-1} (at +70 mV). The rate constants governing open-channel block are $k_{23} = 0{\cdot}025$ μM^{-1} ms^{-1} and $k_{32} = 0{\cdot}01$ ms^{-1} (at −100 mV) or $k_{23} = 0$ (at +70 mV). The concentration dependence of k_{12} and k_{32} are given in the text. The binding constant, K, is assumed to be 7·5 μM, while the open-channel conductance is 30 pS (Na^+ is the charge carrier) at −100 mV and 50 pS (Cs^+ is the charge carrier) at +70 mV. The fractional activation at 100 ms is (a) 0·015, (b) 0·0076, (c) 0·0039, and (d) $5{\cdot}1 \times 10^{-6}$.

Scheme B predicts two kinetic components to each current relaxation. As shown below, a fast ($1/\tau_f$) and a slow ($1/\tau_s$) component are expected and $1/\tau_f$ is much greater than $1/\tau_s$. The calculations show that $1/\tau_2$ provides a good approximation to $1/\tau_f$ under conditions of our experiments. The predictions of scheme B at moderate (3 μM) and intermediate (18 μM) *trans*-Bis-Q concentrations (Fig. 11) do seem to account for the data (Fig. 8) at the two membrane potentials tested. In the case of the higher concentration jump, the calculated relaxation at −100 mV (trace a) shows a fast component ($1/\tau_f = 0{\cdot}53$ ms^{-1}) and an obvious slow component ($1/\tau_s = 0{\cdot}0069$ ms^{-1}), as in Fig. 8*A*; the steady-state occupancy of the burst state is 0·0175. For the smaller agonist concentration jump, however, the calculated inward and outward relaxations (traces c and d) appear to approach the steady state with a rate constant (as in Fig. 8*B*) that is close to $1/\tau_2$ as given by eqn. (6) (i.e. 0·145 ms^{-1} at −100 mV and 0·25 ms^{-1} at +70 mV). The amplitude of the slow component ($1/\tau_s = 0{\cdot}0049$ ms^{-1}) at −100 mV, however, is substantial; but because the rate is so slow, it is not obvious on the time scale examined. Our observation that the two

components have the same sign but very different rate constants suggests that the data can be fit best with a model in which the blocked state, S_3, has a much longer lifetime ($\tau_3 = 1/k_{32}$) than the burst state, S_2; we have therefore used a value of $k_{32} = 0{\cdot}01$ ms^{-1}, although this is probably uncertain by a factor of about 2. The long blocked state has the effect of producing a significant diminution of steady-state current even at very low agonist concentration; the calculated diminution is a factor of five for trace a.

Statistical characteristics of open channels

It was of interest to conduct further statistical analysis of the single-channel records following agonist concentration jumps. From the viewpoint of dose–response relations, the most useful information is obtained at moderate to high agonist concentration. The steady-state distribution of the percentage of time, $P(r)$, during which $r = 0, 1, 2, \ldots n$ channels were open simultaneously was calculated. The results were compared against theoretical binomial or Poisson distributions; but many of the open durations were so brief for $r > 1$ that it was not possible to adequately discriminate between the expected distributions. It was found, however, that $P(r)$ remains time-stationary for up to several hundred episodes in the same membrane patch before obvious deterioration sets in; i.e. in the patch of Fig. 10, $P(1) = 0{\cdot}224 \pm 0{\cdot}100$, 2370 openings. Because this patch contained at least five channels (and probably many more), the results suggest that the fractional activation due to a single channel was quite small. This is, however, partially due to block of open channels by *trans*-Bis-Q and of closed channels by *cis*-Bis-Q.

DISCUSSION

The binding of agonist molecules, evaluated in terms of the slope on double-logarithmic coordinates of steady-state current *vs.* agonist (*trans*-Bis-Q) concentration, was studied at ACh receptors of rat myoballs. The slope of this 'Hill plot' depends on membrane potential; it is closest to two at depolarized potentials, where the fractional receptor activation is smallest, and decreases with hyperpolarization. The Hill coefficient also depends on agonist concentration and was reduced at all membrane potentials at higher agonist concentrations. Currents recorded at hyperpolarized membrane potentials showed evidence of channel block by *trans*-Bis-Q at concentrations $> 2\ \mu$M. Outward currents at depolarized membrane potentials, however, show no evidence of channel block and gave estimates of the Hill coefficient that were closer to two at all agonist concentrations. A simple sequential kinetic model, assuming block of the open conformation by the agonist, was sufficient to predict the macroscopic currents at higher agonist concentrations. In addition, we found that either isomer of Bis-Q can block closed channels.

Critique of the light-flash method

This study shows that the light-flash and patch-clamp techniques are well matched in terms of spatial and temporal resolution. While membrane channels are being recorded electrically, a calibrated bolus of agonist can be produced in < 1 ms and allowed to diffuse away over the next few seconds. The experiment can be repeated

hundreds of times in favourable cases, and there is little indication of receptor desensitization during the time frame under study (~80 ms). The pharmacology of the light-activated agonist seems less favourable. *Trans*-Bis-Q is at least 30-fold less potent at ACh receptors of myoballs than at those of *Electrophorus* electroplaques (Lester, Krouse, Nass, Wassermann & Erlanger, 1980). As a result, half-maximal responses are achieved only at micromolar concentrations where at least two other actions, blockade of both open and closed channels, also occur. This situation severely limits kinetic studies of channel activation at either the macroscopic or single-channel level. The blocking actions may decrease more rapidly than the agonist affinity as the membrane potential is depolarized. The matter is under investigation; more rapid channel gating, however, renders any kinetic study less precise at positive membrane potentials. Channel-blocking effects can also be eliminated in experiments with covalently bound photo-isomerizable agonists (Lester *et al.* 1980; Lester & Chabala, 1984; L. D. Chabala & H. A. Lester, unpublished observations).

Comparison with previous work

Many previous studies have shown that the mechanism of activation of the ACh receptor channel requires the binding of more than one agonist molecule. The current consensus is that the Hill coefficient for powerful agonists approaches two at low agonist concentrations (Colquhoun, 1979), a result confirmed by the present data. Karlin (1967), Colquhoun (1973), and others, however, have shown that the Hill plot, for a variety of kinetic models, would not be expected to be linear and the slope should depend on agonist concentration. It has also been argued that partial agonists would be expected to reduce the Hill slope (Colquhoun, 1973, 1979), and this appears to be the case for decamethonium (Lester, Changeux & Sheridan, 1975), although the channel-blocking properties of decamethonium (Adams & Sakmann, 1978) would have similar consequences. Other work has shown that the Hill coefficient depends on external pH and calcium concentration, and on cholinesterase inhibitors (Peper, Bradley & Dreyer, 1982; Sterz, Dreyer & Peper, 1976). Thus, the Hill coefficient is not in general a constant, and it is usually not an integer.

During a pioneering series of studies on site-directed chemical manipulation of the ACh receptor, Karlin & Bartels (1966) and Karlin (1967) showed that brief exposure to DTT (typically 1 mM for 10 min) reduces the depolarizing response to cholinergic agonists in *Electrophorus* electroplaques. The slope of the Hill plot was reduced from 1·8 to 1·1 (see also Walker, Richardson & McNamee, 1984). In view of the uncertainties concerning dose–response relations measured with voltage jumps in *Electrophorus* electroplaques (Lester *et al* 1975; Lester, Koblin & Sheridan, 1978), these experiments have been extended to other preparations and to more direct electrophysiological measures such as ionic conductance. The reduction of the Hill coefficient after exposure to DTT has been noted in flux measurements on reconstituted *Torpedo* receptors (Walker *et al.* 1984) but not in studies on contraction of chick muscle (Rang & Ritter, 1971), or on conductance in frog muscle (Ben-Haim, Dreyer & Peper, 1975), isolated electroplaques (Lester *et al.* 1980), or rat myoballs in the present study. Although all authors agree that DTT-treated receptors respond poorly, these disagreements on the Hill coefficient suggest that we need to know more about

differences between preparations, agonists, and reducing conditions before deciding whether disulphide reduction alters the functional stoicheiometry of receptor activation by reversibly bound agonists. It seems clearer, however, that a single covalently bound ('tethered') agonist like QBr is sufficient to open the ACh channel after treatment with DTT (Lester *et al.* 1980; Lester & Chabala, 1984; L. D. Chabala & H. A. Lester, unpublished observations).

In agreement with Ben-Haim *et al.* (1975) and Terrar (1978), we also found that the kinetics of the agonist-induced currents were about 2–3-fold faster following treatment of the membrane with DTT. In addition, we found that the steady-state currents were reduced about 3–5-fold under the same conditions. At low agonist concentration, the steady-state conductance for scheme A is proportional to the ratio β'/α'; thus a 2–3-fold increase in α' predicts a similar decrease in the steady-state conductance. The decrease in the steady-state conductance, however, was generally somewhat larger than could be accounted for by the increase in the relaxation rate. It is not clear from our work whether the agonist affinity was lowered or the total number of available channels was reduced, although single-channel experiments with the 'tethered' agonist, QBr, indicate that the open-channel conductance remains near 30 pS at -100 mV after treatment with DTT (Lester & Chabala, 1984; L. D. Chabala & H. A. Lester, unpublished observations).

It has previously been reported that ACh channel kinetics depend on agonist concentration in isolated *Electrophorus* electroplaques (Sheridan & Lester, 1975, 1977) and in frog end-plates (Sakmann & Adams, 1979). Our results, like those of Horn & Brodwick (1980), indicate that macroscopic current relaxations in rat myoballs are composed of a single component at low agonist concentration. Reciprocal relaxation time constants were independent of agonist concentration at low concentrations but increased at higher agonist concentration. The results are consistent with the anticipated influence of agonist concentration on the effective channel opening rate. The dependence of $1/\tau$ on agonist concentration, however, was not studied in detail, because channel block by the agonist is also expected to increase $1/\tau$ for inward currents. In addition, the kinetics of the currents at hyperpolarized membrane potentials became more complicated at higher agonist concentration; they showed two components consistent with open-channel block by the agonist.

Agonist concentration dependence of the Hill coefficient

As expected for receptor activation schemes in which two agonist molecules must bind to open the channel, the Hill coefficient approaches two at low agonist concentrations but decreases at higher agonist concentrations as the available binding sites become saturated. The manner in which the Hill coefficient depends on agonist concentration can offer clues into the mechanism of channel opening; in particular, it can indicate whether the second agonist molecule binds more or less tightly than the first. Because of the pharmacological complications in this study and because of the uncertainties about the molecular mechanism of receptor channel activation, we are not in a position to make a detailed analysis of the dose–response relation. A simple curve-fitting procedure, however, can be used to help interpret the agonist concentration dependence found in the present study. If, for simplicity, it

is assumed that two agonist molecules must bind to open the receptor channel and the isomerization step is included in second binding step, the scheme for receptor activation can be written

$$2A+T \underset{k_{-1}}{\overset{2k_1}{\rightleftharpoons}} AT+A \underset{2k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_2R, \tag{C}$$

where A is the agonist, T is the closed receptor, and R is the open receptor. The equilibrium binding constants are $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$. Since only the relationship between K_1 and K_2 is of interest, no assumption is made regarding whether agonist binding or receptor isomerization is rate limiting. The functional form of steady-state conductance, $g(V_m, \infty)$, is given by the following proportionality:

$$g(V_m, \infty) \propto \frac{A'^2}{A'^2 + mA' + 1}, \tag{7}$$

where the quantity A′ is proportional to agonist concentration and is given by

$$A' = [A]/(K_1 K_2)^{\frac{1}{2}}. \tag{8}$$

The quantity m expresses the ratio of the equilibrium binding constants and is given by

$$m = 2(K_2/K_1)^{\frac{1}{2}}. \tag{9}$$

Because of the statistical factors, values of $m < 2$ suggest positive co-operativity in the microscopic binding constants, while values of $m > 2$ imply negative co-operativity in agonist binding. Since $g(V_m, 0) \sim 0$ for *cis*-Bis-Q solutions (see Methods), the Hill slope, n_H, for eqn. (7) can be approximated by

$$n_H = \frac{\mathrm{d} \log \{g(V_m, \infty)\}}{\mathrm{d} \log (A')}. \tag{10}$$

Each value of m predicts a unique dependence of the Hill slope on $[A]/(K_1K_2)^{\frac{1}{2}}$. Our data, as shown in Fig. 12, are fitted best by values of $m \sim 6$ (with little improvement for $m > 6$). Such values suggest a strong negative co-operativity in the over-all binding of the two agonist molecules (including a possible isomerization step in the second binding); thus, the second agonist molecule must bind much less tightly than the first to open the receptor channel. The half-maximal conductance occurs at the agonist concentration given by

$$[A] = \frac{(K_1 K_2)^{\frac{1}{2}}\{m + (m^2+4)^{\frac{1}{2}}\}}{2}. \tag{11}$$

Thus, the half-maximal conductance would occur at about $6(K_1 K_2)^{\frac{1}{2}}$ for $m \sim 6$, or 100 μM-*trans*-Bis-Q at -100 mV; that value agrees well with estimates based on the present data. For values of $m < 2$, the Hill slope would decrease much more steeply at high agonist concentration than was the case for our data. Although there is some variability in the data of Fig. 12 that is attributable to small sample sizes, there is not enough such that the predictions of positive or negative co-operativity could both overlap the data. Positive co-operativity in agonist binding would also give a half-maximal conductance at a much lower agonist concentration for a given value of $(K_1K_2)^{\frac{1}{2}}$. We note, as well, that preliminary calculations suggest the rising phase

of miniature end-plate currents, at the vertebrate neuromuscular junction, can be fitted better with the assumption of negative co-operativity in agonist binding (Land, Salpeter & Salpeter, 1981; personal communication).

The conclusions regarding the agonist concentration dependence of the Hill slope are not undermined by the existence of open-channel block by the agonist or by the presence of mono- or possibly tri-liganded open receptor channels. Indeed, channel block by the agonist would be expected to reduce the Hill slope, especially in the presence of 20 μM-*cis*-Bis-Q; thus, the Hill slopes at higher agonist concentrations are probably underestimates of the true values. As indicated below, the time integral of conductance due to a population of channels with brief openings is too small to have an impact on the estimates of the Hill slopes. Furthermore, resting receptor desensitization is not a problem in the present experiments. Most of the desensitization that occurs following an agonist concentration jump for negative and positive potentials occurs on a much longer time scale (i.e. seconds, L. D. Chabala & H. A. Lester, unpublished observations); in addition, there was no indication of cumulative desensitization from one trial to the next.

Voltage dependence of the Hill coefficient

The present study also shows that the Hill slope (e.g. the double-log slope) depends on membrane potential between -150 mV and $+80$ mV; it increases with depolarization as the relative receptor activation decreases. In our view this finding does not imply a change in the functional stoicheiometry of receptor activation with membrane potential. It arises instead because one or more of the microscopic rate constants depend on membrane potential; as a result, the half-maximal agonist concentration also depends on membrane potential (cf. Lester *et al.* 1978). A more detailed (and realistic) receptor activation scheme, which includes the isomerization step, is given by the following scheme:

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

where A is the agonist, T represents a closed state, R represents the open state, β is the channel opening rate, and α is the channel closing rate. If most of the voltage dependence of the mean burst length is attributed to $1/\alpha$, the true channel lifetime, calculations like those of Fig. 12, using scheme D and reasonable estimates of the microscopic rate constants, show that there is insufficient voltage sensitivity to account for the observed changes in the Hill coefficient. Ongoing work regarding the voltage sensitivity of the macroscopic transition rates of ACh receptor channels in rat myoballs, however, suggests that β' is also voltage dependent and decreases substantially with membrane depolarization (Chabala & Lester, 1984, 1985). The ratio β'/α' decreases about 10-fold between -100 mV and $+80$ mV (L. D. Chabala & H. A. Lester, unpublished observations). For such a case, the expected changes in the double-log slope would help account for the results. Because β' for scheme D is a function of all the microscopic rate constants except α, it is not clear from the work with macroscopic currents which of those rate constants are voltage dependent; the more likely candidates, however, are the channel opening rate, β, and perhaps the first agonist dissociation rate, k_{-2}. In scheme D, the functional stoicheiometry remains

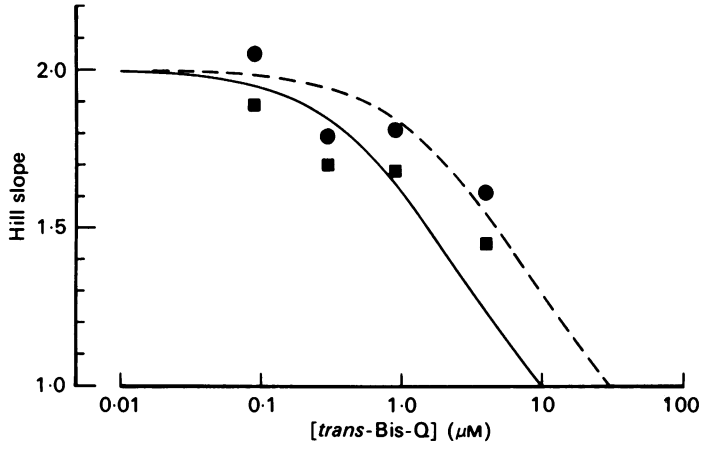

Fig. 12. Concentration dependence of the Hill coefficient. The steady-state conductance was assumed to generate a response that is given by eqn. (7). The Hill slope was calculated from eqn. (10) and was plotted against log ([A]). The curves are plotted for $m = 6$ and for two values of $(K_1K_2)^{\frac{1}{2}}$, 10 μM at -100 mV and 30 μM at $+80$ mV. The data (Table 1) are the least-squares estimates of the Hill slopes at -100 mV (smooth line, filled squares) and at $+80$ mV (dashed line, filled circles). The ordinate for the data is the geometric mean of the [*trans*-Bis-Q] range; this value is 0·17 times the [*cis*-Bis-Q] used for the experiment.

fixed at 2 for all membrane potentials and at all agonist concentrations; nonetheless, the slope of the double-log plot would be less than 2 for particular membrane potentials or agonist concentration domains. It is also closer to 2 at potentials associated with smaller receptor activation. Hence, although the Hill slope may not be intrinsically voltage dependent, the method that is used to estimate it can give rise to an apparent voltage dependence. Thus, a non-integral Hill coefficient would not in itself imply either a change in functional stoicheiometry or an additional population of mono-liganded (or possibly tri-liganded) open receptor channels.

Although our data do not suggest a role for mono-liganded open receptors, such a possibility cannot be ruled out. Single-channel recordings do show a population of brief channel openings, and it has been suggested that this population arises from mono-liganded open channels (Colquhoun & Sakmann, 1981). The time-integral of the conductance due to these channels is so small ($< 5\%$), however, that our experiments lack the sensitivity to test this hypothesis. Models C and D do not predict an excess of brief openings for any reasonable choice of the rate constants; a more accurate molecular model will include additional open (and possibly closed) states. Other single-channel experiments, however, argue against the mono-liganded hypothesis (Lester & Chabala, 1984; Sine & Steinbach, 1984; L. D. Chabala & H. A. Lester, unpublished observations).

Open-channel block by the agonist

The results show that *trans*-Bis-Q begins to block open receptor channels at agonist concentrations > 2 μM at -100 mV. The block is strongly voltage dependent and increases with membrane hyperpolarization. Single-channel recordings at hyperpolarized membrane potentials confirm that bursts are briefer at higher agonist

concentrations, although the mean burst length for a simple receptor activation model such as scheme D would be expected to increase with agonist concentration, since the mean number of openings per burst depends linearly on agonist concentration (cf. Colquhoun & Hawkes, 1981). These results suggest that even if the problems of receptor desensitization could be overcome, a complete analysis of the steady-state dose–response relationship cannot be carried out for Bis-Q at hyperpolarized membrane potentials because of channel block by the agonist; the Hill coefficients would be underestimated at higher agonist concentrations.

Fractional activation of channels at higher agonist concentration

Because the openings were so brief at higher agonist concentration, we were unable to estimate the number of channels in a membrane patch using standard binomial or Poisson statistics. The available data, however, do show clearly that the fractional receptor activation was small. In the experiment of Fig. 10, we observed as many as five superimposed openings; thus the probability that any one channel was open was at most $P(1)/5$, or 0·05. The blockade of both open and closed receptor channels would be expected to reduce the average time spent in the open state; these effects would have combined to diminish the steady-state currents about 5-fold. Thus, a corrected estimate of the fractional activation for a single channel under the conditions of Fig. 10 would be at most 0·25. The actual fractional activation could be several times smaller than this upper limit if the actual number of channels in the patch was substantially greater than five. These rough calculations agree with our conclusion that the whole-cell currents under similar conditions are still at the low end of the dose–response curve, even under the highest concentrations of *trans*-Bis-Q generated by the flashes (10–20 μM).

The authors would like to thank Drs R. Horn and D. P. Corey for suggestions regarding the voltage-clamp techniques used, Dr M. E. Krouse for helpful discussions, and Dr R. E. Sheridan for help in setting up the equipment. Drs B. F. Erlanger and N. H. Wassermann provided the Bis-Q crystals, and the cultured cells were maintained by T. Stevens. This work was supported by fellowships from the Muscular Dystrophy Association of America (L. D. C.) and the Del E. Webb Foundation (L. D. C. & A. M. G.), a Fulbright-Hayes Travel Grant (A. M. G.), and by U.S.P.H.S. grant No. NS-11756.

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