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ACETYLCHOLINE RECEPTOR ACTIVATION BY A SITE-SELECTIVE LIGAND: NATURE OF BRIEF OPEN AND CLOSED STATES IN BC3H–1 CELLS

BY STEVEN M. SINE* AND JOE HENRY STEINBACH†

From the Department of Anesthesiology† and the Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110 and The Salk Institute, P.O. Box 85800, San Diego, CA 92138, U.S.A.

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SUMMARY

1. Single-channel currents were recorded through acetylcholine receptor channels of clonal BC3H–1 muscle cells activated by the curare-like compound, (+)-dimethyltubocurarine (DMT).

2. DMT binds selectively to the two α-neurotoxin-binding sites on these receptors, with apparent dissociation constants differing by about 100-fold (Sine & Taylor, 1981). Receptor channels do not open with DMT bound only to the high-affinity site, but only at DMT concentrations at which both high- and low-affinity sites are occupied.

3. Open-duration histograms are not single exponentials, but are described by the sums of two (or three) exponentials. Both brief- and long-duration openings are observed in the presence of 3 μM-DMT, and are seen at the same relative frequency up to 80 μM-DMT.

4. Long-duration openings are interrupted by brief closures with a mean duration of 50 μs and which occur at a frequency of 50–60 per second of open time. These temporal characteristics closely parallel those of the brief closures observed with the full agonists, acetylcholine, carbamylcholine, and suberyldicholine.

5. Raised concentrations of DMT apparently block open channels in a voltage-dependent fashion.

6. It is concluded that both brief- and long-duration openings arise from receptors with two molecules of DMT bound. Furthermore, brief closures in general do not appear to reflect receptor activation processes. Instead, they seem to arise through entry to a closed state with properties independent of the agonist, but characteristic of open channels.

* Present address: Department of Physiology and Biophysics, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

† Author and address for correspondence.

INTRODUCTION

It is well established that (+)-tubocurarine ((+)-TC) blocks neuromuscular transmission by competing with acetylcholine (ACh) for specific sites on the postsynaptic ACh receptor (AChR) (Jenkinson, 1960). (+)-TC and related antagonists associate with both ACh binding sites, but show selectivity for one site. In particular, (+)-TC and its congener, (+)-dimethyltubocurarine (DMT), are highly selective antagonists, showing greater than a 90-fold difference in binding affinity for the two sites (Neubig & Cohen, 1980; Sine & Taylor, 1981). Recently, however, it has become clear that (+)-TC can also activate AChR in mammalian muscle (embryonic rat muscle: Ziskind & Dennis, 1978; adult rat junctional AChR: Trautmann, 1983; adult rat extrajunctional AChR: B. Sakmann & J. H. Steinbach, unpublished observations; cultured rat myotubes: Takeda & Trautmann, 1984; Morris, Wong, Jackson & Lecar, 1983; cultured human myotubes: Jackson, Lecar, Askanas & Engel, 1982). It does not, apparently, activate AChR on cultured chick myotubes (Takeda & Trautmann, 1984). Therefore, these curariform compounds represent a unique class of cholinergic agonists, possessing a high degree of site selectivity and low intrinsic agonist activity.

Because of these properties, DMT is a potentially powerful ligand for the study of AChR activation processes. For example, ACh activates a single population of AChR channels which adopt two different open states: brief and long duration (Colquhoun & Sakmann, 1981). There is considerable evidence that most open channels have two ACh molecules bound to the receptor (Dionne, Steinbach & Stevens, 1978; Sine & Taylor, 1980), but the nature of brief openings is poorly understood. It has been suggested that brief openings reflect activation of receptors occupied by just one ACh molecule (Colquhoun & Sakmann, 1981; Takeda & Trautmann, 1984), although brief openings have been observed even at saturating concentrations of agonist (Sine & Steinbach, 1984*a*). The high selectivity and known binding function of DMT provide a means of clarifying the number of occupied receptor sites linked to the activation of brief- and long-duration openings.

It is also possible to investigate the nature of brief closed receptor states using DMT. At low concentrations, ACh elicits long-duration openings which appear as bursts of several openings separated by brief closures, described as 'nachschlag' by Colquhoun & Sakmann (1981); these authors suggested that nachschlag reflect brief sojourns of a single receptor in the closed state leading to the open-channel state. Receptor activation processes, therefore, would determine the properties of nachschlag (Colquhoun & Hawkes, 1981). In particular, nachschlag would exhibit temporal properties dictated by the channel opening rate, β, and the dissociation rate of agonist, k_{-2}. The channel opening rate is expected to be substantially lower for the weak agonist, DMT, than for the strong agonist, ACh. Therefore, if nachschlag reflect receptor activation processes, these closures should exhibit different temporal characteristics for DMT than are seen for strong agonists.

Single-channel recording techniques were used to examine the function of the AChR on BC3H–1 clonal muscle cells (Schubert, Harris, Devine & Heinemann, 1974). BC3H–1 cells are particularly suitable for these experiments because ligand binding and tracer ion flux measurements are available for these receptors for many agonists and antagonists (Sine & Taylor, 1979, 1980, 1981, 1982). In particular, DMT shows

a 90-fold difference in binding affinity for the two sites on the AChR of BC3H–1 cells. It is possible, therefore, to examine receptor activation with DMT bound selectively to one site and, at higher concentrations, with a known fraction of receptors occupied by one or two molecules of DMT. Experiments are described which examine the concentration dependence for channel opening by DMT, and the properties of brief closed states.

METHODS

BC3H–1 cells were passaged and prepared for experiments as described previously (Sine & Steinbach, 1984*a*). Cells for experiments were plated on glass cover-slips, maintained in Dulbecco's Modified Eagle Medium plus 0·5 % cadet calf serum (0·5 DME), and treated with proteolytic enzymes 5–6 days before performing experiments. The 0·5 % DME was replaced with fresh medium 1–2 days before performing experiments. Cells were used 13–17 days after plating because agonist-activated currents are relatively simple at these early times (Sine & Steinbach, 1984*a*). Older cells, in contrast, often show agonist-activated currents with multiple conductance classes, and subconductance states are observed.

Single-channel currents were recorded from membrane patches in the 'cell-attached' or 'outside-out' configurations, using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). For all experiments, the extracellular solution contained (mM): KCl, 140; NaCl 5·4; HEPES, 25; $CaCl_2$, 1·8; $MgCl_2$, 1·7, adjusted to pH 7·4 with 10 mM-NaOH. For cell-attached recordings, the pipette was filled with (mM): KCl, 142; NaCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 1·7; HEPES, 9·5; adjusted to pH 7·4 with 5 mM-NaOH. For outside-out patch recordings, the pipette was filled with (mM): KCl, 142; NaCl, 4·5; $MgCl_2$, 2·0; EGTA, 1; HEPES, 9·5; adjusted to pH 7·4 with 5 mM-NaOH. (+)-dimethyltubocurarine iodide (DMT) was kindly provided by Ely Lilly (Indianapolis, IN). For outside-out patch experiments, the concentration of DMT was changed by adding a measured volume of 10 × concentrated solution of DMT dissolved in extracellular solution and waiting 5 min for mixing. This method of drug exposure was found to be well suited for use with these fragile outside-out patches. A temperature of $11 \pm 0{\cdot}5$ °C was maintained using a Peltier temperature-controlling device. The bath volume was 0·7–1·0 ml, measured after each concentration series.

Data were recorded and analysed as described previously (Sine & Steinbach, 1984*a*). Briefly, single-channel currents were recorded on FM analog tape (15 in. s^{-1}) and replayed at lower tape speed for analysis. Analog records were filtered (eight-pole Bessel filter with effective cut-off frequencies given as the −3 db point), and digitized at intervals of 25–50 μs. The entire record was processed using an interrupt-driven sampling routine provided by Dr F. J. Sigworth. Digitized records were scanned in consecutive groups of 4096 points, and any group containing a possible opening was stored on magnetic disks. A possible opening was defined as any deviation from base line of approximately one-third the full open-channel current, or more. This definition is less restrictive than the definition of a transition used in subsequent analysis. Transitions were detected in the digitized data using a threshold crossing procedure with threshold set at half the full open-channel current. Transitions had to persist for a specified time to be accepted, usually three or four sample points. Data were then stored as a series of triplets consisting of open durations, closed durations and current amplitudes.

Open-duration and closed-duration histograms were plotted as probability density functions. Histograms were fitted by the sum of exponential components. Components were fitted sequentially, from the slowest to the fastest, using a non-linear least-squares routine. The histogram was displayed, and the operator selected a minimum duration beyond which the data appeared to show only a single exponential component. The data were grouped into intervals of varying duration which contained at least five entries, and a single exponential was fitted to the grouped data. Two methods were used for determining the best fitting exponential, either minimizing the sum of the squared deviations or χ^2. The methods gave indistinguishable results; all values presented were obtained by minimizing the sum of the squared deviations. After fitting the component, its contribution was subtracted from the histogram and the next fastest component was fitted.

For some patches the opening frequency was too low to record enough events to generate a smooth histogram. Therefore we also generated summed histograms of data from different patches recorded under identical conditions of DMT concentration, membrane potential and temperature.

RESULTS

Qualitative features of DMT-induced single-channel currents

Fig. 1 shows the essential features of single-channel currents induced by a low concentration of DMT (3 μM). The records reveal the occurrence of channel openings

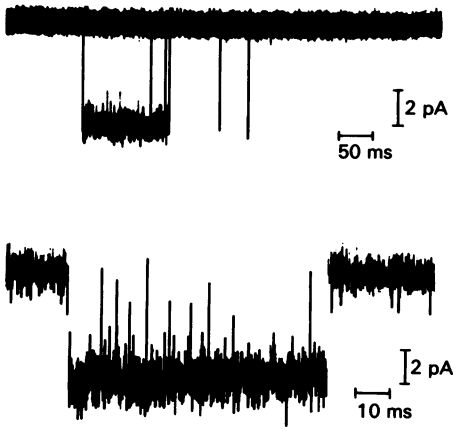


Fig. 1. Oscilloscope traces of single-channel currents recorded from a cell-attached patch in the presence of 3 μM-DMT, -130 mV, 11 °C. Top panel, several consecutive sweeps are superimposed to show the appearance of brief- and long-duration openings, low-pass filtered at 3000 Hz. Bottom panel, an especially long burst of openings is displayed at higher resolution, low-pass filtered at 8000 Hz. Note the occurrence of several brief closures.

with both brief and long durations. Brief openings appear as isolated events, whereas long-duration openings often occur as bursts of several openings separated by brief closures. As described below, these brief closures exhibit the temporal characteristics of nachschlag seen with classical agonists (Colquhoun & Sakmann, 1981; see Table 1) and they appear to reflect full closure of the channel. DMT elicits openings at a low frequency, often as low as 0·1–0·3 s^{-1}. Under similar conditions, 100 nM-ACh induces openings at a rate of 1–5 s^{-1}; occasionally several channels are open simultaneously. The single-channel conductance of DMT-activated channels is 36 ± 7 pS ($n = 35$). Under these conditions, the single-channel conductances for channels activated by full agonists are 38 ± 3 pS (ACh), 34 ± 3 pS (carbamylcholine) and 38 ± 4 pS (suberyldicholine). Thus, DMT activates single-channel currents with properties similar to those activated by ACh, although it appears to be a very ineffective agonist.

Single-channel currents were examined to determine the number of conductance states associated with receptor activation by DMT. The mean current amplitude is plotted against the channel open time in Fig. 2*A*. The amplitude–duration plot

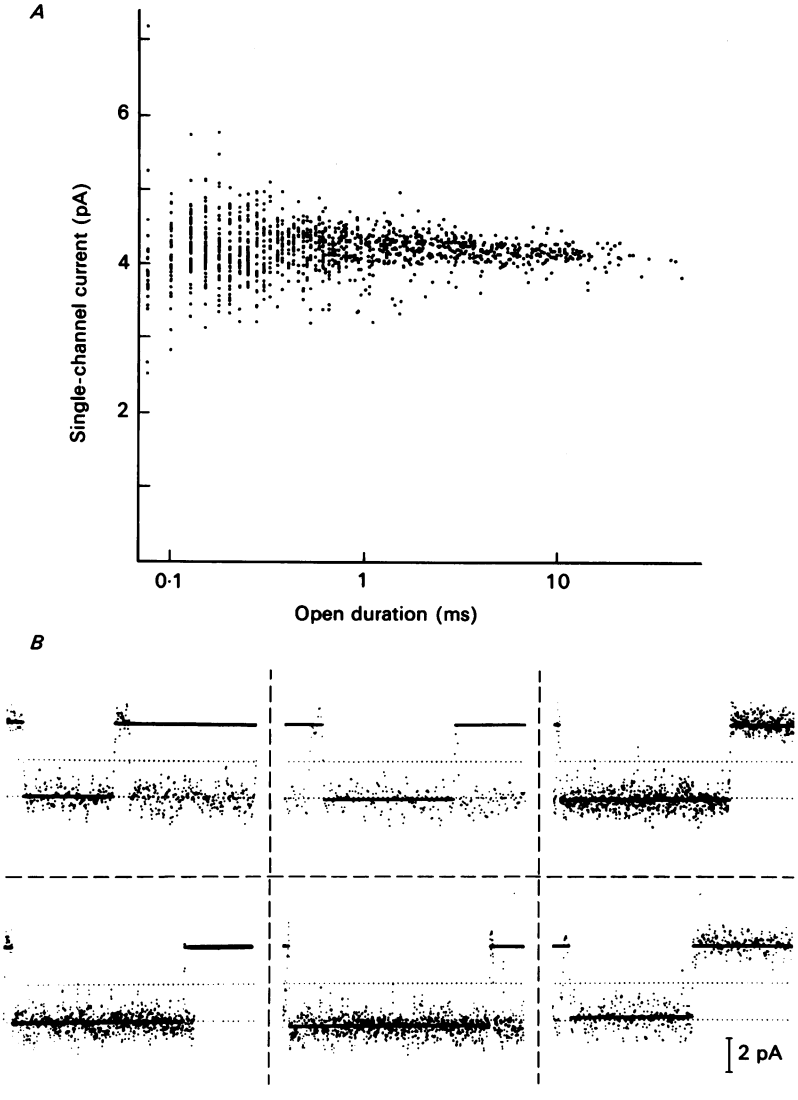


Fig. 2. Upper panel, a plot of mean current amplitude *vs.* the channel open time for currents recorded from a cell-attached patch exposed to 3 μM-DMT, −100 mV, 11 °C, low-pass filtered at 8000 Hz. There is only one amplitude class of channels present. Lower panel, examples of detected brief closures recorded in the presence of 3 μM-DMT, −130 mV, 11 °C. The record was filtered at 8000 Hz and digitized at 25 μs intervals. The continuous lines show the mean base-line and open-channel current levels, and the dashed line shows the threshold for detection. Closures were detected which crossed threshold for at least three consecutive sample points. Note that the detected closures reach the mean base-line current level.

appears as a single cloud of points, showing that brief- and long-duration openings have the same unit conductance. Examples of brief closures are shown in Fig. 2*B*. These high-resolution measurements reveal that detected closures return to the mean base-line current level. Therefore, opening and closing transitions result in a single step change in conductance.

DMT binds at two distinguishable sites on the AChR of BC3H–1 cells, with dissociation constants of 0·3 and 28 μM (Sine & Taylor, 1981). Therefore, the concentration dependence for AChR activation was assessed in a preliminary fashion by recording currents from cell-attached patches with DMT concentrations of 0·3–100 μM in the recording pipette. Channel-opening events were rarely observed at concentrations below 1 μM, but were seen regularly above 3 μM. These findings are

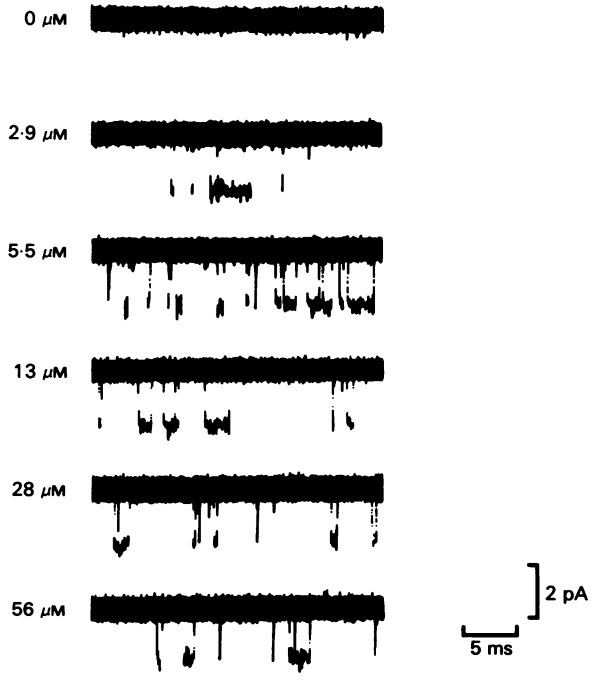


Fig. 3. Oscilloscope records from one outside-out patch exposed to the indicated concentrations of DMT at −50 mV, 11 °C. Thirty consecutive seconds of each record were superimposed, low-pass filtered at 2000 Hz. Channel-opening events are not observed in the absence of DMT or with 0·3 μM-DMT but appear following the addition of 2·9 μM-DMT.

qualitatively consistent with the idea that DMT triggers channel openings when it is bound to both high- and low-affinity sites of the AChR channel. The following experiments examine this concentration dependence in detail using outside-out patches.

Concentration dependence for DMT-induced activation

Outside-out patches were formed in order to expose the same population of receptors to different concentrations of DMT. Records are illustrated from one patch exposed to several concentrations of DMT (Fig. 3). Channel-opening events are not observed in the absence of DMT, and are very rare at concentrations at which only the high-affinity site is occupied (0·2–0·4 μM). Opening events appear, however, at DMT concentrations at which both the high- and low-affinity sites would be occupied.

Fig. 4 shows a comparison of the frequency of channel activation and the

occupation function for DMT binding to the AChR of BC3H–1 cells (Sine & Taylor, 1981). Receptor channels activate at concentrations above about 2 μM, which results in occupation of 86 % of high-affinity sites and 6 % of low-affinity sites. Clearly, AChR activation is closely associated with occupation of both high- and low-affinity sites by DMT.

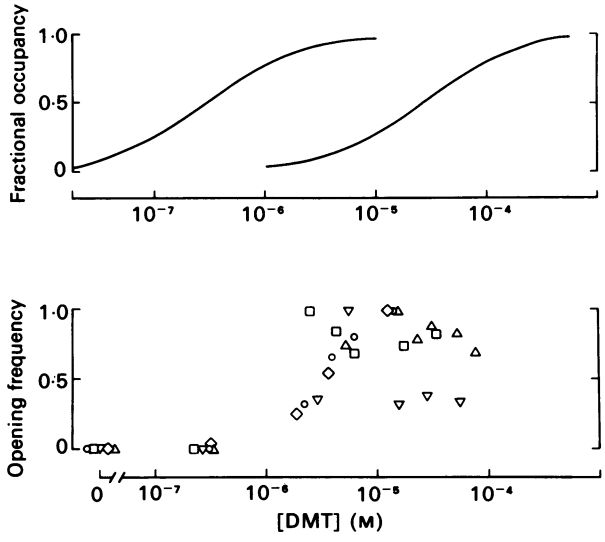


Fig. 4. Top panel, a plot of the predicted fraction of receptor sites occupied by DMT, shown separately for the high-affinity (left curve) and low-affinity (right curve) sites. The concentration dependence was calculated using the measured dissociation constants for DMT binding to AChRs on BC3H–1 cells; $K_A = 0{\cdot}3$ μM, $K_B = 28$ μM (see Sine & Taylor, 1981). Bottom panel, the relative opening frequency was measured at the indicated concentration of DMT for five different outside-out patches (one symbol per patch), −50 mV, and 11 °C. The opening frequency is calculated relative to the maximum frequency observed for each patch (range, 0·1–0·4 s^{-1}). Channel openings were not observed below 1 μM-DMT but were detected at concentrations above 2 μM.

This concentration dependence suggests that the rate of channel activation should increase with increased occupancy of the low-affinity site, which has an apparent dissociation constant of 28 μM (see Fig. 4). The opening frequency increases as expected between 2 and 10 μM, but remains constant or decreases at concentrations above 10 μM. We do not know the reason for this lower-than-expected opening frequency at higher DMT concentrations. It is likely that high concentrations of DMT cause long-lived block of the channel (see below). In addition, it is our impression that patches suffer a loss of receptors during the prolonged periods required for these experiments (about 90 min for the experiment in Fig. 3). For these reasons we will not attempt to interpret quantitatively the concentration dependence of the opening frequency or the distribution of long closed times between independent openings. The major, qualitative, point in Fig. 4 is that no activity is seen with concentrations of DMT at which the majority of ACh receptors will have one (and only one) ligand-binding site occupied.

Concentration dependence of open durations

Open-duration histograms were analysed from several outside-out patches for which there were relatively large numbers of openings. The histograms are not well described by a single exponential distribution, but are fitted well by the sum of two exponentials. Fig. 5 shows histograms of open durations obtained from one patch in the presence of 5·5 and 33 μM-DT. For both concentrations brief- and long-duration

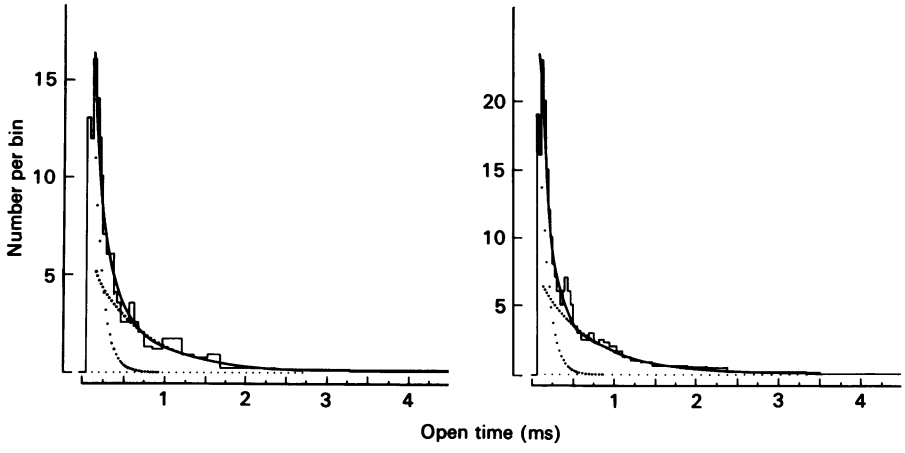


Fig. 5. Open-duration histograms from outside-out patches exposed to the indicated concentrations of DMT at −50 mV, 11 °C. Records were low-pass filtered at 3400 Hz and digitized at intervals of 50 μs. Open and closed transitions were detected which lasted 150 μs or longer, using a detection threshold midway between the mean base-line and open-channel currents. Each histogram is fitted by the sum of two exponentials (continuous curve) with components shown by the lower dotted curves. For 5·5 μM (left panel), recording time of 627 s, 216 events: $t_0 = 1{\cdot}2$ ms, fraction = 0·44; $t_1 = 199$ μs, fraction = 0·56. For 33 μM (right panel), 620 s record, 273 events: $t_0 = 1{\cdot}3$ ms, fraction = 0·51; $t_1 = 194$ μs, fraction = 0·49.

openings are distinguished clearly with well separated time constants and approximately equal areas. These time constants did not change appreciably over the concentrations of DMT used, at −50 mV. As described below, high concentrations of DMT shorten the duration of long openings, apparently by blocking the open channel. Channel block, however, is not apparent in these measurements because the forward blocking rate is reduced at the depolarized potential of −50 mV.

The ratio of brief to long openings was examined over a wide range of DMT concentrations to determine the concentration dependence for eliciting both classes of opening. Brief and long events are classified as openings shorter or longer than a specified discriminator time. This discriminator time was established as the duration at which the brief and long probability density functions were equal (Jackson *et al.* 1982). Although two exponential components are always needed to fit the open-duration histograms, it was not always possible to estimate the two time constants because many records contained only 50–150 events. Therefore, the discriminator time was estimated from seven records which contained relatively large numbers of

events (200–450 entries). The mean discriminator time from these records (0·6 ms) was used to classify events for all five patches recorded under identical conditions (11 °C, −50 mV) at concentrations of 1·8–81·6 μM. Fig. 6 shows that the fraction of long openings is virtually constant at 0·55 over the entire concentration range. This constant fraction provides additional evidence that brief and long openings result from receptors with both sites occupied by DMT.

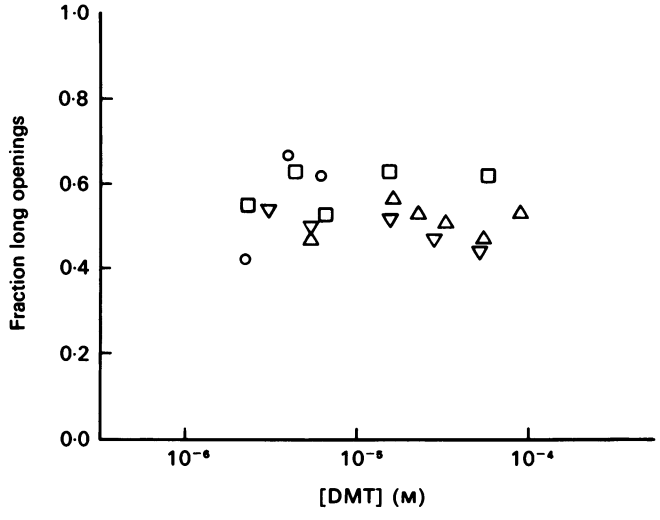


Fig. 6. The fraction of long-duration openings is plotted against the concentration of DMT (same primary data as Fig. 4). A discriminator time of 0·6 ms was used for each record to classify openings as long or brief. The fraction of long openings appears constant over a wide range of DMT concentrations.

We assume that the rates for receptor activation are independent of time within the record. To test the assumption of stationarity, each record was divided into equal time intervals and the number of brief and long events was counted in each interval. Openings were again classified using the discriminator time described in the previous section. Fig. 7 shows the results from a representative record which exhibits stationarity. Brief and long events occur at a constant rate across the record. In all five outside-out patch experiments examined, this analysis indicated that the kinetic processes appear stationary for generating brief- and long-duration openings.

The records were examined further by dividing each record into relatively short time intervals (50 ms–5 s) and counting the number of events in each class in each interval. The observed number of intervals containing 0, 1, 2,... events were compared with the predictions of a Poisson distribution. The data were described well by the Poisson distribution, providing additional evidence that the records are stationary, and that events occur both randomly and independently. Hence there is no coupling apparent between events.

Departures from the Poisson prediction are expected when channel openings occur as bursts of openings separated by brief closures, as shown in Fig. 1. At −50 mV, openings rarely occur as bursts because the rate of nachschlag occurrence is low

compared to the rate for channel closing. At −100 mV, however, long-duration openings often occur as bursts due to the increased rate of nachschlag occurrence and the decreased rate of channel closing (see following section).

High-resolution measurements

It is necessary to record for prolonged periods at hyperpolarized potentials (−100 mV) in order to perform a detailed analysis of open and closed durations with high temporal resolution. Cell-attached patches are usually stable for up to 1 h at potentials between −70 and −130 mV, and exhibit low background noise (0·5–0·6 pA r.m.s. at 8000 Hz). Therefore, the high resolution measurements described below were obtained using the cell-attached patch configuration.

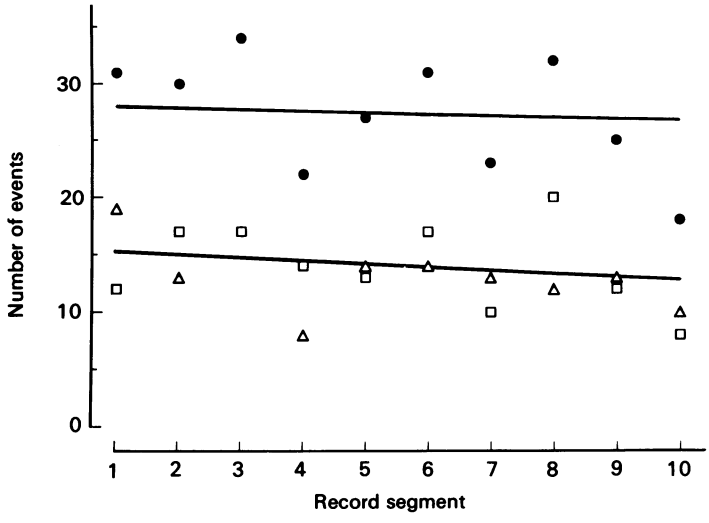


Fig. 7. The record in Fig. 5, 33 μM, was tested for stationarity by counting the number of channel openings in each of ten equal time intervals (62 s each). The discriminator time of 0·6 ms was used to classify openings as long or brief: total events, 273 (●); brief events, 133 (△); long events, 140 (□). The data were analysed using a linear regression fit. The slope in events/interval, is: total events, −0·48; brief events, −0·28; long events, −0·21. These slopes indicate a slight decrease in the frequency of events across the record, but are not significantly different from zero.

Analysis of closed-duration histograms

Closed-duration histograms were analysed from records obtained from cell-attached patches in the presence of 3 μM-DMT at a potential of −100 mV. The closed-duration histogram is described by the sum of exponential terms: a major brief-duration component, a major long-duration component, and a small but detectable intermediate component. For the histogram shown in Fig. 8, the brief component has a time constant of 57 μs and represents 11 % of the area of the histogram. These brief closures occur exclusively between long-duration openings and are clearly similar to the nachschlag seen with classical agonists (Table 1). The long-duration component has a time constant of 0·9 s, contributes 86 % of the area, and represents closed periods

between independent openings. The intermediate component is difficult to examine quantitatively because it has such a small area (< 3 %). None the less, an intermediate component is often observed, with a time constant of about 1 ms.

Closed-duration histograms were summed from several records in order to increase the number of entries in brief-duration bins. Fig. 8 shows the summed histogram from seven patches recorded under identical conditions (3 μM-DMT, −100 mV, 11 °C). The

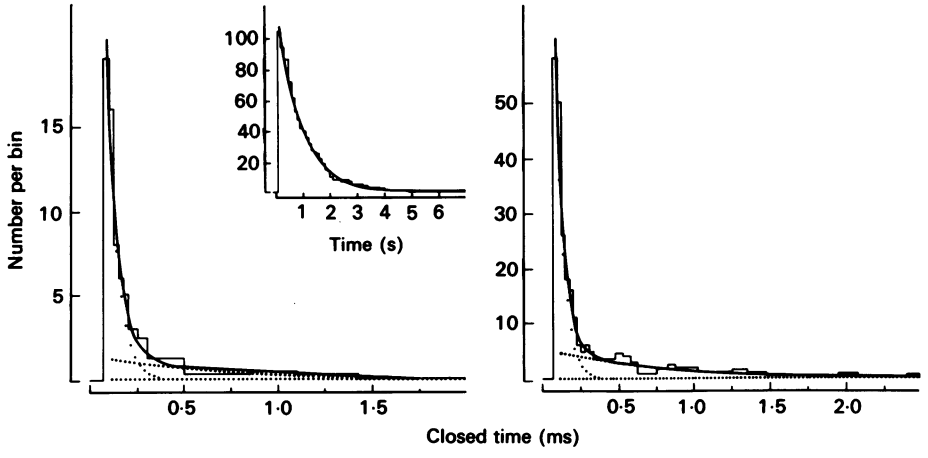


Fig. 8. Closed-duration histograms constructed from currents recorded from cell-attached patches in the presence of 3 μM-DMT, −100 mV, 11 °C. Records were low-pass filtered at 7800 Hz, digitized at intervals of 25 μs, and closures were detected which lasted 75 μs or longer. Left panel: the data from a single patch are shown on two time scales to illustrate brief- (main panel) and long-duration (inset) closures. The histogram is fitted by the sum of three exponentials (upper continuous curve) with the contributions shown by the lower dotted curves. The time constants and fractions of the total area are: t_0 = 905 ms, fraction = 0·856; t_1 = 0·767 ms, n_1 = 0·031; t_2 = 57·5 μs, fraction = 0·113; record length = 1055 s; 1270 events. Right panel: a closed-duration histogram summed from data recorded from seven cell-attached patches with 3 μM-DMT, −100 mV, 11 °C. The histogram is fitted with the sum of three exponentials (upper continuous curve) with contributions shown by the lower dotted curves: t_0 = 1586 ms, fraction = 0·822; t_1 = 1·11 ms, fraction = 0·039; t_2 = 59·8 μs, fraction = 0·139; 5196 events in histogram.

summed histogram also exhibits a clear brief component with a time constant of 58 μs and an area of 14 %. A small intermediate component is evident again with a time constant of 1 ms and an area of about 4 %. A slow component is present with a time constant of several seconds but cannot be interpreted because of the variable frequency of openings in each record.

To summarize, these closed-duration histograms reveal that low concentrations of DMT activate channel openings which exhibit brief transitions to a low conductance state. These brief closures have a time constant of about 50 μs, which is similar to the duration of nachschlag seen with classical agonists.

Brief closures, or nachschlag, were examined further by computing the number of nachschlag per burst of openings and the number per second of open time. The number of nachschlag was estimated from the area of the brief component fitted to the closed-duration histogram. A burst was defined as any sequence of openings separated

by closed periods shorter than 1·5 ms, and the number of bursts estimated from the area of the slow component of the burst-duration histogram (see next section). In the presence of 3 μM-DMT, there were 0·48 nachschlag per burst, occurring at a rate of 60 per second of open time. Table 1 compares these values with those obtained for the classical agonists, ACh, carbamylcholine and suberyldicholine. The number

TABLE 1. Comparison of nachschlag

Compound	n	τ nachschlag[a] (μs)	Nachschlag rate[b] (s^{-1})	τ burst[c] (ms)	Nachschlag/burst[d]
DMT (3 μM)	(3)	58·6±7·2	70±29	7·8±1·3	0·63±0·28
	(7)	53·0	59	6·9	0·48
ACh (100 nM)	(4)	48·3±7·8	48±24	51·5±8·9	2·45±1·27
	(4)	44·8	51	52·7	2·73
Carb. (2 μM)	(6)	49·9±9·3	59±30	23·5±6·0	1·27±0·39
	(6)	53·1	55	20·9	1·24
Sub. (50 nM)	(2)	60·9±15	49±24	58±20	2·63±0·29
	(2)	54·4	50	52·4	2·60

Two rows of parameters are presented for each agonist: DMT, ACh, carbamylcholine (Carb.) and suberyldicholine (Sub.) The upper row is the mean (±S.D.) for the data from n patches. The lower row gives values from analysis of a single summed histogram of data from n patches. All data obtained at −100 mV, 11 °C.

[a] The time constant for the major brief-duration closed component.

[b] The ratio of the number of brief closures to the total open time in the record.

[c] The time constant of the slowest component of the burst-duration histogram.

[d] The ratio of the areas of brief closures to long-duration bursts.

of nachschlag per burst is 2–5-fold lower for DMT than for the agonists. Surprisingly, nachschlag are observed at the same rate during channel openings for both DMT and the classical agonists.

It is possible that nachschlag reflect receptor activation processes. The approach of Colquhoun & Sakmann (1981) was used to estimate the channel opening rate, β, and the ligand dissociation rate, k_{-2}. At low ligand concentration, the number of nachschlag per burst is approximately β/k_{-2} and the time constant for nachschlag is $(\beta+k_{-2})^{-1}$. These relationships provide estimates for β and k_{-2} of 6000 and 13000 s^{-1}, respectively. Although the estimate for β is lower than this analysis yields for agonists (11000–14000 s^{-1}, it is far too large to be consistent with the low frequency of openings observed with 3 μM-DMT.

We calculated the expected frequency of channel openings using the equations of Colquhoun & Hawkes (1981) for a four-state activation scheme with these estimates of β and k_{-2} and the dissociation constants estimated from measurements of DMT binding. These calculations predict that 3 μM-DMT should elicit openings at a frequency of 300 s^{-1} per receptor. In general, the observed frequency is 0·1–1·0 s^{-1}, more than 300 times lower than is expected for a single receptor. This estimate of β becomes even more unlikely considering that each patch contains many receptors.

High-resolution measurements of channel-open durations

DMT-induced single-channel currents were examined further to gain information on the voltage dependence of brief- and long-duration openings and to provide a

preliminary description of channel block. High-resolution measurements were obtained from cell-attached patches at DMT concentrations of 3, 30 and 100 μM at 11 °C.

Fig. 9 shows a representative open-duration histogram of currents recorded from a cell-attached patch in the presence of 3 μM-DMT at −100 mV. Brief- and long-duration openings are evident with well separated time constants of 200 μs and

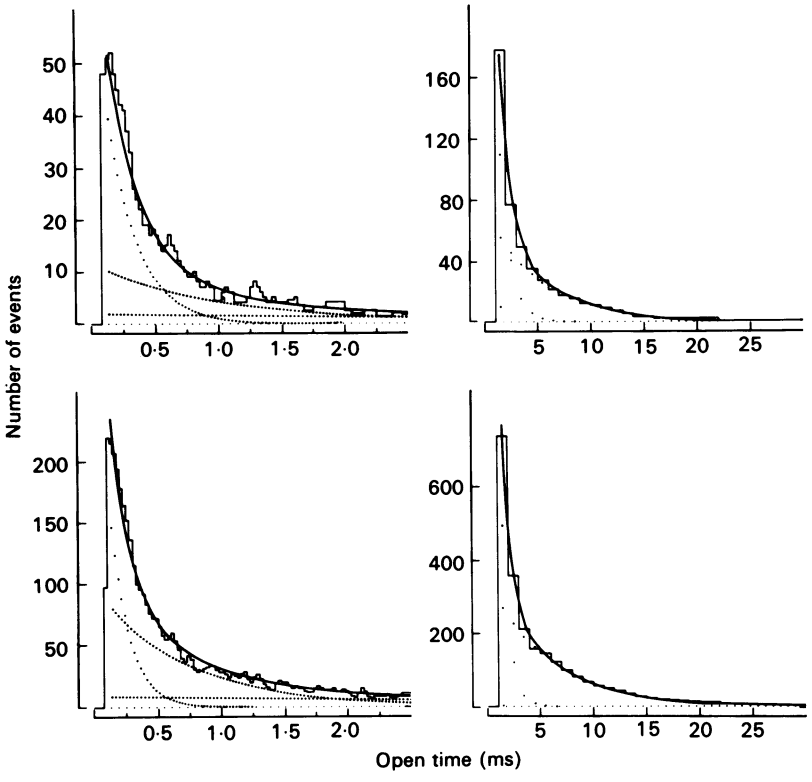


Fig. 9. Open-duration histograms recorded from cell-attached patches with 3 μM-DMT, −100 mV, 11 °C. Upper two panels: the histogram shown is from one patch (the closed-duration histogram is shown in Fig. 8). Lower two panels: the histogram presents the sum of data from seven patches recorded under identical conditions. Each histogram is shown on two time scales, the left quadrant brief-, the right quadrant longer-duration openings. Each histogram is fitted with the sum of three exponentials (continuous curves) with components shown by the lower dotted curves. For the upper panel: t_0 = 5·20 ms, fraction = 0·25; t_1 = 1·01 ms, fraction = 0·30; t_2 = 238 μs, fraction = 0·44, 1270 events. For the lower panel: t_0 = 5·86 ms, fraction = 0·30; t_1 = 0·70 ms, fraction = 0·39; t_2 = 147 μs, fraction = 0·31, 5597 events.

6·5ms. In addition, an intermediate component is observed consistently at this more hyperpolarized potential, with a time constant of 0·7 ms and an area of up to 25% of the histogram. The open-duration histogram summed for seven patches also exhibits these three well separated components (Fig. 9). These results show that brief- and long-duration openings may be distinguished clearly and reveal an additional intermediate class of opening at hyperpolarized potentials.

A burst was identified as any sequence of openings separated by closures shorter than 1·5 ms. The burst duration, then, is the total time of the open periods plus the intervening brief closed periods. Bursts are distinguished clearly in each record because the time between bursts is typically 1–10 s. Fig. 10 shows the burst-duration histogram from currents recorded in the presence of 3 μM-DMT at −100 mV. The burst-duration histogram appears similar to the corresponding open-duration histo-

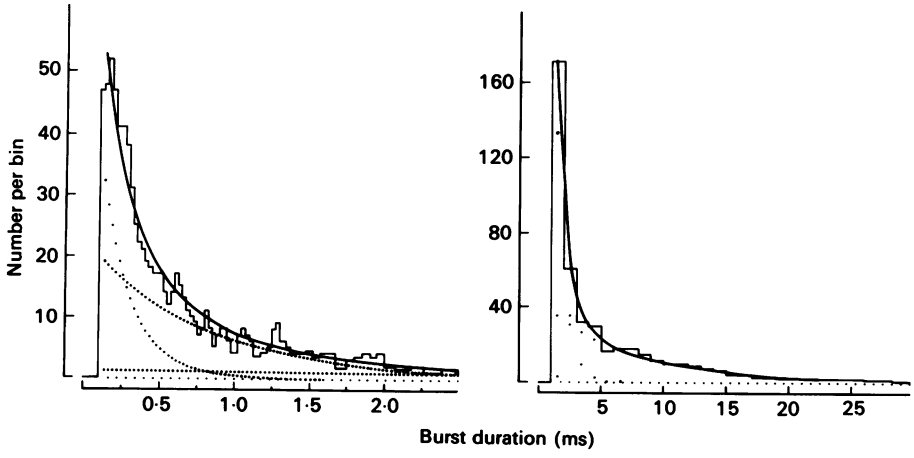


Fig. 10. Burst-duration histogram generated from currents recorded from a cell-attached patch: 3 μM-DMT, −100 mV, 11 °C. (the open-duration histogram is shown in Fig. 9). High-resolution records were concatenated using a minimum closed duration of 1·5 ms. The histogram is shown on two time scales: the right panel shows bursts lasting longer than 1 ms. The histogram is fitted with the sum of three exponentials (upper continuous curves) with individual components shown by the lower dotted curves. t_0 = 6·78 ms, fraction = 0·22; t_1 = 0·751 ms, fraction = 0·46; t_2 = 191 μs, fraction = 0·33; 1164 events.

gram (Fig. 9), but exhibits some quantitative differences. The long-duration time constant is prolonged in the burst-duration histogram, while the brief and intermediate components show little change. These results are consistent with the observation that brief closures occur primarily between long-duration openings. The number of bursts in the record was estimated from the area of the long-duration component and this value used to compute the number of nachschlag per burst (Table 1).

Brief- and long-duration openings were observed in the presence of 30 μM-DMT at a potential of −100 mV (Fig. 11). The fraction of brief openings is not reduced between 3 and 30 μM, yet the binding measurements predict a 10-fold decrease in the ratio of singly to doubly occupied receptors. This maintained excess of brief openings is consistent with the results from outside-out patches which demonstrate the strong association between doubly occupied receptors and the activation of both brief and long openings. The time constant of brief openings is relatively unchanged between 3 and 30 μM, whereas the long-duration time constant is reduced about 3-fold. This reduced open duration is consistent with the concept that raised concentrations of DMT induce block of the open channel.

Voltage dependence of brief and long openings

Brief- and long-duration openings were examined at several membrane potentials in the presence of either 3 or 30 μM-DMT. The long-duration time constant was

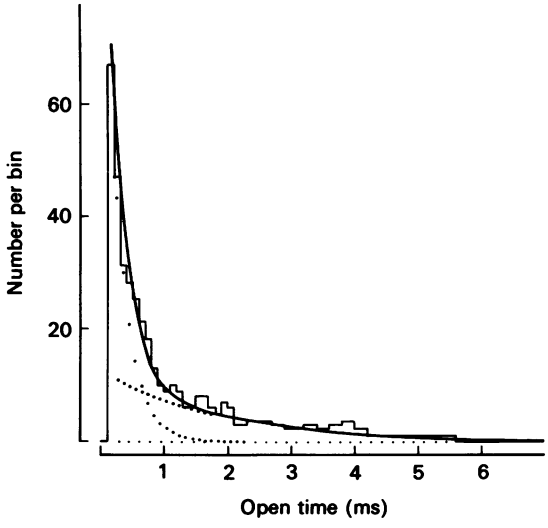


Fig. 11. Open-duration histogram of currents elicited by 30 μM-DMT, -100 mV, 11 °C. High-resolution records were analysed as described in Figs. 8 and 10 and openings longer than 100 μs are shown. The histogram is fitted with the sum of two exponentials (continuous curve) with components shown by the lower dotted curves: $t_0 = 1{\cdot}91$ ms, fraction = 0·45; $t_1 = 270$ μs, fraction = 0·55; record length = 1826 s; 439 events.

estimated from the fit to the slowest component of the open-duration histogram, and the brief-duration time constant from the fit to the fastest component. In the presence of 3 μM-DMT, long-duration openings are prolonged at more negative potentials, increasing e-fold per change of 60 mV (Fig. 12). In contrast, at 30 μM, long-duration openings show a slightly reduced open time at more negative potentials. These voltage dependencies demonstrate that DMT shows a potential-dependent blocking action. It is evident, therefore, that channel block may be minimized by maintaining a low membrane potential, as we have described for the studies of concentration dependence with outside-out patches.

Open and closed durations were examined further to provide a preliminary description of the forward and backward blocking reactions. Fig. 13 shows that the reciprocal of the long-duration mean open time increases linearly with DMT concentration. The slope of the line reveals an apparent forward blocking rate constant of 10^7 M^{-1} s^{-1}, at -70 mV and 11 °C. The forward blocking rate is quite voltage-dependent, as shown in Fig. 12. The differences between open times at 3 and 30 μM-DMT give a voltage dependence for the forward blocking rate of an e-fold increase per hyperpolarization of 90 mV. Data similar to those in Fig. 13, at concentrations of 3 and 30 μM-DMT and potentials of -100 and -130 mV, provide a second estimate for the voltage dependence of the forward blocking reaction of e-fold per hyperpolarization of 83 mV.

Closed-duration histograms were examined at high concentrations of DMT (30 and 100 μM) in an effort to identify the unblocking processes. In most experiments closed-duration histograms were well described by a single exponential component with a long time constant, probably reflecting periods between independent channel openings. In some experiments two brief-duration components were observed with

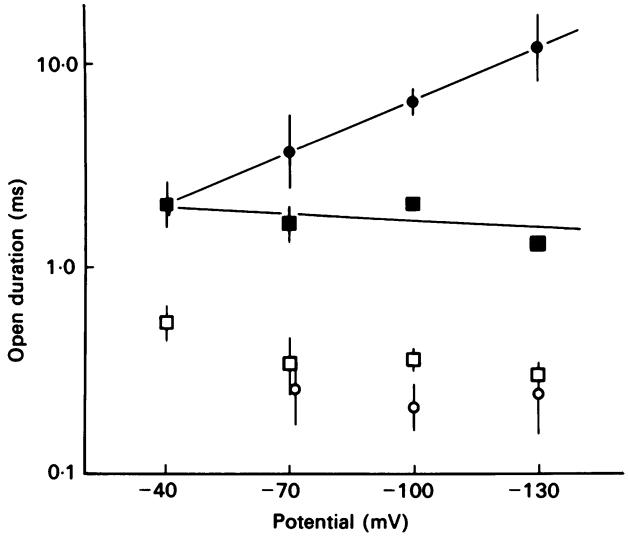


Fig. 12. Voltage dependence of brief- and long-duration openings. The mean time constants for openings are shown, ±1 S.D. Those for long-duration openings are shown as filled symbols for 3 μM (●) and 30 μM (■)-DMT. Time constants for brief openings are plotted as open symbols for 3 μM (○) and 30 μM (□)-DMT. Currents were recorded from cell-attached patches at the potentials shown at 11 °C. Histograms were fitted with the sum of two or three exponentials, with the slowest component corresponding to long-duration openings and the fastest component corresponding to brief-duration openings. An intermediate component was observed only with 3 μM-DMT and was distinguished clearly at −130 mV and in about half of the records at −100 mV. The time constant of the intermediate component increased at more negative potentials, but the data were not extensive enough to examine its voltage dependence in detail.

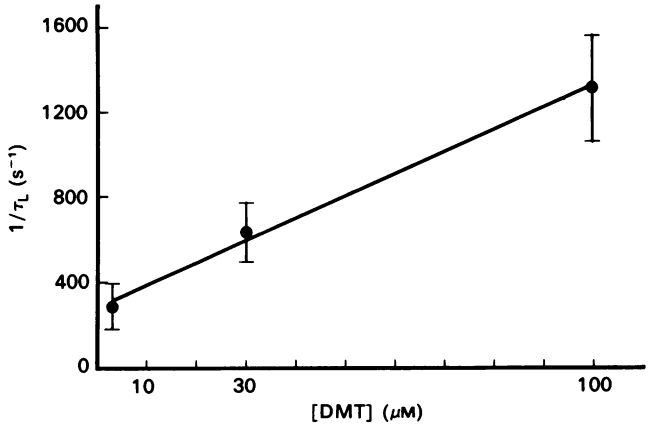


Fig. 13. Concentration dependence of the apparent forward blocking rate for DMT. Currents were recorded from cell-attached patches and the reciprocal of the long-duration time constant is plotted against the specified concentrations of DMT (mean ± S.D.). The line shown has a slope of $1{\cdot}0 \times 10^7$ M^{-1} s^{-1} (−70 mV, 11 °C).

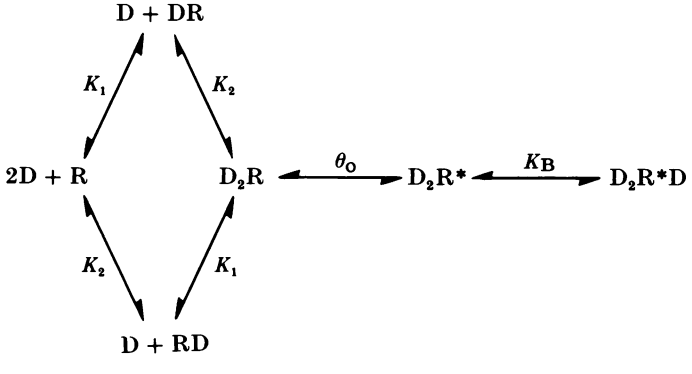
small areas, corresponding to the brief and intermediate closures seen with low concentrations of DMT. Brief closures were rarely observed at these high concentrations, presumably because the rate of block becomes large relative to the rate of occurrence of these closures. The duration of channel-blocking events, therefore, was not apparent in these closed-duration histograms. We conclude that either the blocked state lasts a long time (seconds) or that unblocking may proceed through closed receptor states.

DISCUSSION

We have examined activation of ACh receptors by the curariform antagonist, DMT. Receptor channels do not open with DMT bound selectively to the high-affinity site, but activate when both high- and low-affinity sites are occupied. Both brief- and long-duration openings are observed at low DMT concentrations, and occur at the same relative frequency over a wide range of DMT concentrations. Long-duration openings often occur in bursts of several openings separated by brief closed periods. The brief closures exhibit temporal characteristics very similar to those of nachschlag observed with full agonists. These close similarities suggest that nachschlag, seen with both strong agonists and with DMT, do not reflect the receptor activation steps β and k_{-2}. Rather, nachschlag apparently reflect an additional closed state with properties independent of the agonist used to open the channel. Raised concentrations of DMT apparently block the open channel in a voltage-dependent fashion.

BC3H–1 cells provide the key to the design and interpretation of these experiments. Measurements of receptor–ligand binding and agonist-induced Na influx demonstrate that receptors of BC3H–1 cells have two α-toxin sites linked to receptor activation, and antagonists block activation by binding at either α-toxin site (Sine & Taylor, 1980, 1981). DMT binds to both α-toxin sites, but exhibits high affinity for one site and blocks agonist-induced Na influx by associating with the high-affinity site. These studies have shown that receptor activation closely parallels occupancy of the two α-toxin sites, and they have defined conditions in which DMT may be used to occupy one site selectively.

Sine & Taylor (1981) analysed their binding data under the assumption that these processes did not occur. Therefore, it can reasonably be asked whether the data on DMT binding are distorted by the additional processes of channel opening and open-channel block described in this paper. The following scheme can be used to predict the proportion of free α-neurotoxin sites as a function of DMT concentration, where D is DMT and R is a receptor with a closed channel, R* is a receptor with an open channel, and D_2R^*D is a receptor with an open-blocked channel:



The equilibrium dissociation constants (K_D s) for the DMT binding sites are K_1 (high affinity) and K_2, the opening equilibrium constant is $\theta_0 = \beta/\alpha$ and the blocking dissociation constant is K_B. The available toxin-binding sites are then:

$$\text{fraction free} = \frac{1}{2}\left[\frac{2 + D/K_1 + D/K_2}{1 + D/K_1 + D/K_2 + (D^2/K_1K_2)(1 + \theta_0(1 + D/K_B))}\right], \quad (1)$$

where D is the concentration of DMT. This equation reduces to the form used by Sine & Taylor (1981) if $\theta_0 = 0$. Our data indicate that channel opening (and hence open-channel block) only occur when both sites are occupied (concentrations above 2 μM). Since $K_1 \ll K_2$, at these concentrations ($D \geqslant 10\ K_1$) eqn. (1) simplifies to:

$$(\text{fraction free})' = \frac{1}{2}\left[\frac{1}{1 + D/K_2 + (D/K_2)(\theta_0(1 + D/K_B))}\right]. \quad (2)$$

Sine & Taylor (1981) used an analogous equation to fit the titration of the low-affinity binding site:

$$(\text{fraction free})'' = \frac{1}{2}\left[\frac{1}{1 + D/K_2}\right]. \quad (3)$$

Clearly, the presence of channel opening and block will distort estimates of K_2 made using the simple predicted binding function (compare eqns. (2) and (3)). However, the amount of distortion depends on θ_0 and D/K_B. An estimate of K_B must be tentative, as the unblocking rate is not known, but K_B might be 10–200 nM at −70 mV and 11 °C. Sine & Taylor's (1981) binding data were acquired using depolarized cells, at 4 °C. K_B must increase because of the voltage dependence of the forward rate, perhaps to 1 μM. Referring to eqn. (2), it is clear that the term D/K_B is comparable to, and probably larger than, 1 when $D > 10K_1$. This implies that if θ_0 is appreciable, eqn. (3) cannot describe the data since the denominator of eqn. (2) will contain a significant term in D^2. In short, the initial rate of α-neurotoxin binding at high concentrations of DMT should decrease more sharply than a simple titration curve, given a value of K_B comparable to K_2 and θ_0 appreciable compared to 1. The actual binding data (Sine & Taylor, 1981), are well described by eqn. (3); if anything, the data curve slightly more shallowly. Hence, the binding data suggest that θ_0 must be small, and that the titration curves are probably not seriously distorted.

A second, more circumstantial, piece of evidence is provided by examining the ability of (+)-TC and gallamine to inhibit the initial rate of α-neurotoxin binding after partial occupation of toxin-binding sites by α-neurotoxin (Sine & Taylor, 1980). The initial rate of α-neurotoxin binding was determined at 21 °C as a function of ligand concentration with control cells, and after 64–67 % of the α-neurotoxin sites had been randomly occupied by α-neurotoxin. No change was observed in either the (+)-TC or gallamine binding curves. If it is assumed that receptors with one binding site occupied by α-neurotoxin do not open (Sine & Taylor, 1980), and hence cannot suffer open-channel block, the lack of change in the two curves suggests that there is no serious distortion due to channel opening and block. The partial toxin occupation experiments again suggest that θ_0 must be relatively small.

These arguments in favour of the idea that β/α is small require that block be relatively effective ($K_B \leqslant K_2$, see eqns. (2) and (3)). The present data suggest that block is indeed quite efficacious. Hence, the binding data suggest that θ_0 is small, and that the apparent dissociation constants estimated from the binding data are reasonably accurate.

It is likely that DMT does not desensitize receptors. Weiland & Taylor (1979) reported no change in the affinity of agonists for *Torpedo* receptor after exposure to DMT. Sine & Taylor (1979) reported that there is no difference in the ability of (+)-TC to reduce the initial rate of α-neurotoxin binding to BC3H–1 cells when α-neurotoxin and (+)-TC were added simultaneously, as compared to a 20 min pre-exposure to (+)-TC. Finally, in our experiments using DMT on outside-out patches, we saw no evidence for grouping or clustering of openings over the concentration range studied

(the physiological data could be complicated by channel block). Over-all, these data suggest that DMT is relatively ineffective, both as an agonist and as a desensitizing ligand.

DMT offers several advantages over classical agonists for the study of receptor activation processes. Measurements of the binding of strong agonists are difficult to interpret because agonist-activated receptors adopt several states with high probability: resting, active and two desensitized states (Steinbach, 1980; Sakmann, Patlak & Neher, 1980). Since each state contributes to the measured binding function, there is substantial uncertainty in predicting the concentration dependence of receptors with one or two agonists bound. In contrast, measurements of DMT binding may be interpreted more readily as the association of a ligand with two sites on the ACh receptor with different affinity. DMT does not induce desensitization, and appears to be an ineffective agonist with a channel opening rate, β, much lower than that for classical agonists.

Our results clarify the nature of the open states associated with AChR activation. It has been suggested that brief openings result from receptors with one agonist molecule bound, whereas long openings are associated with receptors occupied by two agonists (Colquhoun & Sakmann, 1981; Takeda & Trautmann, 1984). The present results show that DMT does not activate receptor channels when only the high-affinity site is occupied, but triggers both brief- and long-duration openings when both sites are occupied. The present results complement our previous observation that a single receptor may adopt both brief- and long-duration open states at saturating concentrations of agonist (Sine & Steinbach, 1984*a*). Although it is not certain that both brief and long open states are strictly comparable between agonists and DMT, it is clear that a receptor may enter at least two open states with both sites occupied by the activating ligand. We have not obtained any evidence for receptor activation by only one bound agonist molecule.

These observations also provide insight into the nature of one class of brief closed state, commonly known as nachschlag. Colquhoun & Hawkes (1977) first suggested that, at low agonist concentrations, receptors might open and close repeatedly before the agonist could dissociate. Therefore the distribution of brief closures would depend on the channel opening rate, β, and the agonist dissociation rate, k_{-2}. Several research groups have observed nachschlag, and have estimated β and k_{-2} from nachschlag (Colquhoun & Sakmann, 1981, 1983; Dionne & Leibowitz, 1982; Leibowitz & Dionne, 1984; Sine & Steinbach, 1984*a*). These experiments with DMT test the hypothesis that this class of brief closings reflects the receptor activation steps of channel opening and agonist dissociation.

Surprisingly, with DMT there is a major brief closed time with a mean duration of about 50 μs. Analysis of these closures provides estimates of β and k_{-2} of 6000 and 13000 s^{-1} respectively, values which appear exceptionally high given the low efficacy of DMT relative to ACh. Incorporated into a four-state activation scheme, these rate constants predict a far greater frequency of channel opening than is observed experimentally. Interestingly, nachschlag occur at the same rate during channel openings for DMT and for all three classical agonists examined. These results suggest that nachschlag do not reflect receptor activation processes for DMT.

It is important to consider whether serious errors exist in the burst frequency predicted by the estimates of β and k_{-2} from brief closings and the apparent dissociation constants from binding data. Two factors can readily be envisioned which could distort the estimate. The first is that K_D values from binding are seriously in error. Since it is likely that there are about 100 receptors in a patch, based on α-neurotoxin binding (Patrick, McMillan, Wolfson & O'Brien, 1977), only about 1 in 10000 receptors could be active to produce the observed low frequency of openings. This implies that K_2 from binding would have to be inaccurate by this amount, which seems unlikely (see above).

The second possibility is that channel block could reduce the number of receptors with available channels. In a strict linear scheme for open-channel block, every blocking episode is terminated by an unblocking event and hence, if a series of open-channel dwells induced by block are not recognized but are thought to reflect independent activations, block would increase the apparent number of openings. The situation is more complicated if it is assumed that blocked channels can close, because the unblocking rate from closed channels is completely unknown. An extreme case would be one in which the blocking drug is completely trapped in closed channels (e.g. chlorisondamine: Lingle, 1983). In this case a significant fraction of the total channels could be drawn into a non-activatable form. No quantitative predictions can be made, since so many parameters are unknown. However, qualitatively this hypothesis and similar, less extreme, cases of closure of blocked channels would most likely produce a decrease in channel-opening frequency with hyperpolarization as the forward blocking rate increases. The data in the present experiments with 3 μM-DMT showed no change or an increase in event frequency with hyperpolarization (see also Morris *et al.* (1983), for experiments using (+)-TC). It might be expected, as well, that pre-exposure to (+)-TC would produce a shift in occupancy curves if a significant fraction of the receptors were drawn into a long-lived closed-blocked form, whereas no shift is seen (Sine & Taylor, 1979).

A related possibility is that closed-channel block could reduce the number of receptors with available channels. The present data do not directly address this possibility. However, the ability of DMT to inhibit carbamylcholine-induced Na ion fluxes in BC3H–1 cells can be described by occupancy of the high-affinity site (K_1 = 300 nM: Sine & Taylor, 1981), so it is unlikely that closed-channel block is more efficacious than open-channel block. If closed-channel block occurred with a K_B of 50 nM, at 3 μM-DMT the fraction of available channels would be reduced to 0·016, still not enough to make the predicted and observed opening frequencies agree. In the case of (+)-TC acting on frog junctional AChR, Colquhoun, Dreyer & Sheridan (1979) have suggested that closed-channel block is unlikely to be a major phenomenon.

These arguments against the possibility that channel block seriously reduces the rate of observed open-channel episodes are all circumstantial. Over-all, though, it seems unlikely that block could result in such a low frequency of events that estimates of β and k_{-2} made from the properties of brief closures could be consistent with the observed low frequency.

A final possibility is that DMT, and indeed any agonist, could bind to the ligand-binding site in two modes: 'activating' and 'non-activating'. In this case, it would not be appropriate to predict the opening frequency using apparent dissociation constants for total site occupancy. So long as the activating mode of binding was quite rare compared to the non-activating mode, it would be possible to have simple titration curves for site occupancy, and a very low frequency of seen openings. There is no evidence supporting this possibility, and by the nature of the hypothesis such evidence would be difficult to obtain. On the other hand, the possibility cannot be ruled out at present.

If nachschlag do not reflect receptor activation processes, what do they reflect? Nachschlag clearly represent a closed state which is closely coupled to the open state. The number of nachschlag per burst varies between agonists according to the mean burst duration, but nachschlag occur at a relatively constant rate during channel openings for all agonists at a given potential. These observations suggest that nachschlag result from a process intrinsic to the open channel, which is independent of the agonist used to activate the channel. Nachschlag, then, would reflect the transition of active receptors to a short-lived closed state. This unimolecular reaction has a forward rate constant of 60 s^{-1} and a backward rate of about 20000 s^{-1}.

About 3 % of the closures seen using DMT as agonist have an intermediate duration of about 1 ms. The nature of intermediate closures remains unknown.

Raised concentrations of DMT apparently block the open channel in a voltage-dependent fashion. The general properties of channel block have been examined largely in order to define conditions in which block is minimized. The forward rate for block is 10^7 M^{-1} s^{-1} at -70 mV, similar to the blocking rates reported for (+)-TC (Colquhoun *et al.* 1979; Takeda & Trautmann, 1984), quaternary local anaesthetics (Neher & Steinbach, 1978), and suberyldicholine (Sine & Steinbach, 1984*b*). The forward blocking rate increases with hyperpolarization about e-fold per 90 mV, close to values reported for (+)-TC (Colquhoun *et al.* 1979; Takeda & Trautmann, 1984). The unblocking process could not be distinguished, suggesting that it is either very slow or can proceed through closed receptor states, also in agreement with observations with (+)-TC. Although block was minimized in experiments with outside-out patches by using a low applied potential of -50 mV, block may contribute to the lower-than-expected opening frequency observed at DMT concentrations above 10 μM.

At hyperpolarized voltages an intermediate duration class of openings is observed. At high DMT concentrations intermediate openings were not distinguished, probably due to the effects of channel block on long-duration openings. The origin of this class of openings is unknown, but its presence could affect estimates of the proportion of brief-duration openings. If intermediate openings are present but not distinguished clearly, the mean duration of long openings would be underestimated, and the duration of brief openings over-estimated. The majority of intermediate openings would be counted as brief at low DMT concentrations, so the presence of undetected intermediate openings would increase the apparent proportion of brief openings. At higher concentrations, channel block, which reduces the duration of long openings preferentially, would cause more intermediate openings to be counted as long openings. Therefore, at high concentrations the presence of intermediate openings would probably decrease the measured proportion of brief openings. Since a constant proportion of brief openings is seen over a wide concentration range, either there is no distortion introduced by intermediate openings or the fraction of brief openings actually increases with concentration.

Takeda & Trautmann (1984) studied ACh receptor activation by (+)-TC using primary cultures of rat myotubes. Their observations differ from ours in two respects. First, they found that (+)-TC activated a low-or partial-conductance channel. No comparable currents were seen on these BC3H–1 cells. Secondly, they reported that brief duration openings were more frequent at lower (+)-TC concentrations. In contrast, receptor channels on BC3H–1 cells do not open when exposed to DMT concentrations at which only the high-affinity site would be occupied. However, their observations were made by comparing different cell-attached patches at various concentrations. The present studies of the concentration dependence of brief and long openings were made on outside-out patches exposed to a series of DMT concentrations, and were conducted at 11 °C to slow receptor kinetics and -50 mV to minimize channel block. The major advantage in the present study, however, is that the concentration dependence for DMT binding to the ACh receptor on BC3H–1 cells is known (Sine & Taylor, 1981).

A linear four-state activation scheme can account for most properties of macroscopic currents activated by ACh:

$$2A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A + AR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^* \qquad \text{(A)}$$

In scheme (A), two agonist molecules (A) bind to a receptor with a closed channel (R), then the channel opens (R*). Patch-clamp data have revealed some new features of ACh receptor function, but it is not clear how the patch-clamp data are to be interpreted. These results obtained using a weak agonist indicate that additional receptor states need to be added to scheme (A), in agreement with previously reported results with ACh and carbamylcholine (Sine & Steinbach, 1984*a*). This is shown in scheme (B):

$$2A + R \rightleftharpoons A + AR \rightleftharpoons A_2R \rightleftharpoons A_2R^* \underset{b}{\overset{f}{\rightleftharpoons}} A_2R' \qquad \text{(B)}$$

$$A_2Q \rightleftharpoons A_2Q^*$$

The brief-duration closed state (seen with all agonists examined) is unlikely to result from transitions from A_2R^* to A_2R and back to A_2R^*. Instead, it appears to result from entry into A_2R', a closed state to the right of A_2R^* (see also Auerbach & Sachs, 1984). Furthermore, there is no evidence that brief-duration openings reflect the activation of ACh receptors with only one ligand-binding site occupied by agonist. Instead, both brief and long duration openings appear to result from receptors with the same number of bound agonist molecules; with DMT this number is two. Apparently, brief-duration openings reflect a short-lived open state, A_2Q^*, with an associated closed state, A_2Q. It is not known how the receptor states A_nR and A_nQ are interconnected. There are two implications from these results. At least for these cells the ACh receptor has more available functional states than had been expected. It is not known what physiological significance these new states have. Further, these results indicate that it is not necessarily straightforward to associate dwell times seen in patch-clamp data with steps in hypothetical reaction schemes, even for a relatively well studied membrane channel.

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