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EFFECTS OF CHLORPROMAZINE AND PHENCYCLIDINE ON MOUSE C2 ACETYLCHOLINE RECEPTOR KINETICS

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SUMMARY

1. Patch-clamp techniques were used to record acetylcholine- (ACh) activated single-channel currents in cell-attached membrane patches from myotubes of the mouse cell line, C2.

2. The effects of the phenothiazine derivative chlorpromazine (CPZ) and of the hallucinogen phencyclidine (PCP) on ACh-activated single-channel properties were studied under conditions where both compounds are positively charged (pH 7·2).

3. The single-channel conductance was unaffected by either CPZ or PCP at concentrations ranging from 10 to 500 nM.

4. 10–200 nM-CPZ and PCP led to shortened mean burst times.

5. CPZ and PCP effects on mean burst times were voltage independent and did not vary in a simple linear manner with concentration.

6. 10–200 nM-CPZ and PCP did not reduce channel opening frequencies, suggesting that the fraction of non-conducting state (occupied, blocked or desensitized) favoured at equilibrium was not significant at these concentrations.

7. On the other hand, concentrations of CPZ and PCP higher than 300 nM did lead to depressed channel opening frequencies. In addition, we observed that, at these concentrations, the shortened burst duration reverses to the longer values found at lower effector concentrations.

8. The effects of CPZ and PCP on ACh-activated single-channel kinetics are interpreted in terms of current models of ACh-receptor structure and conformational transitions.

INTRODUCTION

The non-competitive blockers of the permeability response to acetylcholine (ACh) constitute a large and rather heterogeneous collection of pharmacological agents which provide potentially useful tools for the structural identification of the ion channel associated with the ACh receptor and of the mechanisms responsible for the

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regulation of its opening by ACh (review in Adams, 1981; Changeux, 1981; Heidmann, Oswald & Changeux, 1983). A large set of electrophysiological measurements based on 'noise' analysis, voltage-jump relaxation, and single-channel recordings have led to the proposal that these compounds could transiently enter and 'plug' the ion channel and thereby sterically inhibit ion translocation (reviewed in Adams, 1981). Such a mechanism would account for the voltage dependence and linear variation with concentration of the blocking effect found with several of the non-competitive blockers studied (Adams, 1976, 1977; Neher & Steinbach, 1978). Alternatively, other (and even often the same) compounds have been postulated to inhibit ion transport by an allosteric mechanism through the stabilization and/or acceleration of a transition(s) toward a closed (desensitized) receptor-channel conformation(s) (Neher & Steinbach, 1978; Koblin & Lester, 1979; Katz & Miledi, 1980; Changeux, 1981; Oswald, Heidmann & Changeux, 1983).

In parallel, *in vitro* experiments carried out with ACh receptor-rich membranes and/or purified receptor protein reconstituted into lipid bilayers disclosed the existence of at least two main categories of binding sites on the ACh-receptor molecule (reviewed in Heidmann *et al.* 1983; Changeux, Devillers-Thiéry & Chemouilli, 1984): (1) high-affinity sites, sensitive to histrionicotoxin and present as a unique copy per receptor pentamer and (2) low-affinity sites, insensitive to histrionicotoxin, much more numerous and lipid-dependent. Binding of the non-competitive blockers to these sites in general elicits a cascade of conformational transitions and conversely binding of fluorescent (Grünhagen & Changeux, 1976; Grünhagen, Iwatsubo & Changeux, 1977) or radioactive (Cohen, 1978; Krodel, Beckman & Cohen, 1979; Oswald *et al.* 1983) non-competitive blockers has served to monitor the allosteric transitions of the receptor protein (reviewed in Changeux *et al.* 1984). Two of them, the phenothiazine chlorpromazine (CPZ) and the hallucinogen phencyclidine (PCP) because of their high reactivity and selectivity have been extensively used to label the ACh-receptor polypeptides in the diverse transient or equilibrium conformations of the molecule (Oswald & Changeux, 1981; Oswald *et al.* 1983; Heidmann & Changeux, 1984). However, their effects on the receptor channel properties have been explored mostly in the micromolar range of concentration (Koblin & Lester, 1979; Albuquerque, Tsai, Avonstam, Eldefrawi & Eldefrawi, 1980; Anwyl & Narahashi, 1980; Magleby & Pallota, 1981) distinct from the range of affinity (100 nM) of the high-affinity site assayed directly by biochemical measurements in *Torpedo marmorata* (Heidmann *et al.* 1983; Oswald *et al.* 1983; Heidmann & Changeux, 1984).

In view of these findings, we decided to investigate the actions of CPZ and PCP at nanomolar levels on the ACh-activated single-channel currents by cell-attached patch-clamp method (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with C2 mouse myotubes. Some of these results have appeared in a preliminary form (Ribera, Trautmann, Pinset & Changeux, 1985).

METHODS

Culture preparation

We recloned a stock of the mouse myogenic cell line C2 (gift of Dr David Yaffé, Weizmann Institute, Rehovot, Israel). Clone 7 (passages 2–5) was used for all experiments. Myoblasts were plated onto collagen-coated 35 mm plastic culture dishes (Falcon, Oxnard, CA, U.S.A.) at a density

of $10^3/cm^2$. Cultures were grown at 37 °C in a humidified atmosphere containing air and 5% CO_2 in Coons modified F12 medium supplemented with 20% fetal calf serum (Gibco, Paisley, Scotland) and 1 μM-dexamethasone (Sigma). After four days, they reached confluence but were still mononucleate. Fusion was induced by replacing the Coons F12 medium with Dulbecco modified Eagle's medium (DMEM; KC Biological Inc., Lenaxa, KS, U.S.A.) containing 4·5 g glucose/l, 10 μg bovine insulin/ml (Sigma) and 5 μg human transferrin/l (Sigma; Pinset & Whalen, 1985). Under these conditions multinucleated myoblasts began to appear 16 h later. Culture media were changed to fresh media every 48 h. Cultures were used one to eight days after the switch to DMEM medium.

The C2 myotubes had resting potentials between −60 and −70 mV as measured by patch electrodes in the whole-cell recording mode under current clamp (Hamill *et al.* 1981). In a few cases, the reversal potential was determined directly as −3 mV (by using successively in the same cell, the cell-attached mode to measure ACh-induced currents at various pipette potentials, and the whole-cell recording mode to measure the resting potential). The value of the reversal potential of ACh-activated channels was later used as the −3 mV reference for the patch potential in the cell-attached configuration.

Spontaneous contractions of C2 myotubes were rarely observed.

Single-channel recording

The experiments were performed at room temperature (20–23 °C). Before use, the culture medium was removed from a C2 preparation, and the dish was immediately washed with the bath solution (in mM: NaCl, 140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 11; HEPES, 5, pH 7·2). The culture dish was then mounted on an inverted phase-contrast microscope (Zeiss) and viewed at a maximum magnification of 256 times. A Narashige 3-D hydraulic micromanipulator was used to control the patch electrode position.

Electrodes were pulled from soft glass (Assistent microhaematocrit) coated with Sylgard to approximately 100 μm from their tips which were then fire polished. Electrodes were filled with a solution similar to bath but without glucose. Electrodes had resistances of 3–6 MΩ when measured in the bath.

Single-channel currents were recorded using a patch-clamp amplifier (List EPC – 7) from tight seal (50–100 GΩ) cell-attached patches (Hamill *et al.* 1981). Data were stored on tape (Racal FM/4) at a 5 kHz band width.

Data analysis

Data were analysed with an LSI 11/23 DEC computer. Single-channel recordings were digitized with a sampling interval of 0·2 ms and filtered at 1 kHz with an 8 pole Bessel Filter (Frequency Devices). Amplitude histograms of single-channel currents were made as described by Takeda & Trautmann (1984). Briefly, for several blocks (512 points, approx 0·1 s) of digitized data, the amplitudes of the points from a base line (determined by eye) were measured and used to construct a histogram giving the number of occurrences at a given amplitude. The position of the peaks give the various amplitude levels reached during channel openings. The ordinates give the *relative* cumulative durations spent at each amplitude level.

Kinetic measurements were done on recordings showing a maximum of two simultaneous open channels. When two ACh-activated channels were open simultaneously, the computer tabulated such events by assigning randomly the first closure to either the first or the second opening.

ACh-activated channel kinetics were characterized by several parameters: burst time, percentage of open time due to a kinetic class of channels, number of transient closures per burst. The calculations of these values are explained below.

(*i*) *Burst time.* We observed that channel openings were occasionally interrupted by brief closures, as first reported by Colquhoun & Sakmann (1981). In constructing our histograms of open durations, we chose to exclude these transient closures (briefer than 3 ms see Fig. 1*B* and Results). We refer to the open time thus measured as the burst time. Open (and closed) duration histograms were fitted to a single exponential using a least-squares method on a semilogarithmic representation of the data. Such fits would yield mean open (or closed) time.

(*ii*) *Percentage of open time due to a kinetic class of channels.* Open duration histograms had fast and slow components. We estimated the percentage of open time due to rapid channels by determining the number of openings contributing to each component and multiplying that value by the mean burst time associated with that component. The number of channels contributing to

each component was determined from the area under the corresponding exponential extrapolated to zero duration. This allows unresolved brief events to be taken into account. The amount of open time due to the fast component was divided by the total open time (sum of open times due to fast and slow components).

(*iii*) *Number of transient closures per burst.* The total number of transient closures was estimated from the area under the exponential fitting the brief closed time distribution (Fig. 1*B*). This value was divided by the number of bursts that occurred in the same recording, to give the number of transient closures per burst.

Chemicals

AChCl (Fluka), CPZ (Sigma) or PCP was added to the pipette solution. PCP was synthesized by C. Hirth (Strasbourg, France). ACh was used at a concentration of 200 nM. CPZ and PCP were used at a concentration of 10–500 nM. The purities of CPZ and PCP were determined by thin layer chromatography on silica gel 60 F (Merck). In a solvent system of ethylacetate:butanol:ethanol:ammonium hydroxide (70:15:10:1), CPZ migrated as a single spot with a relative band speed coefficient (R_f) of 0·48. PCP chromatographed as one spot with an R_f coefficient of 0·55 in a solvent system of methanol ammonium hydroxide (99:1).

RESULTS

ACh-activated single-channel currents in C2 myotubes

ACh-activated single-channel currents were recorded from cultured mouse C2 cells in the presence of 200 nM-ACh in the cell-attached configuration. All the patches studied presented the channel activity (Fig. 1*A*1) but such events were not observed if ACh was not included in the pipette solution. During the recording (Fig. 1*A*1), the patch membrane was held at a potential 100 mV hyperpolarized from rest giving a transmembrane potential of −155 mV (see Methods). Under these conditions, the current amplitudes were distributed in a Gaussian fashion centred around a value of 4·9 pA (Fig. 1*A*2), indicating that there was one conductance class of channels in the patch. (In some patches a larger conductance (48 pS) class of channels was observed; these channels will not be discussed here.) The small peak around 9·8 pA corresponded to the current produced by two simultaneously occurring channel openings.

Closed time distributions are shown in Fig. 1*B*. Two distinct kinetic components are evident. The slow component (time constant, τ = 33 ms) corresponds to the time between individual channel openings. The fast component, shown in detail in

Fig. 1. Kinetic properties of ACh-activated single-channel currents in a C2 myotube patch. *A*1, single-channel currents recorded from a cell-attached patch. The pipette solution contained 200 nM-ACh in Ringer solution. Patch potential, −155 mV. Data filtered at 1 kHz; 2, amplitude histogram of ACh-activated currents. Transmembrane potential, −155 mV. The large peak occurs at 4·9 pA. A smaller peak at 9·8 pA corresponds to the current produced by two simultaneous channel openings. *B*, closed time histograms for the same C2 patch. Transmembrane potential, −155 mV. 1, a single exponential was fitted to the slow component of the closed time distribution. The time constant (τ) is 33 ms, and corresponds to the time between single-channel openings. 2, the first component of the same closed time distribution shown at an expanded scale. The time constant is 0·49 ms and reflects the transient closures of open channels. *C*, open time histograms for the sample patch. Transmembrane potential, −155 mV. 1, the distribution of open times was fitted with two exponential components. Most of the openings were fitted with an exponential having a time constant of 32 ms. 2, the fast component of the same open time distribution shown at an expanded scale. The time constant is 0·64 ms.

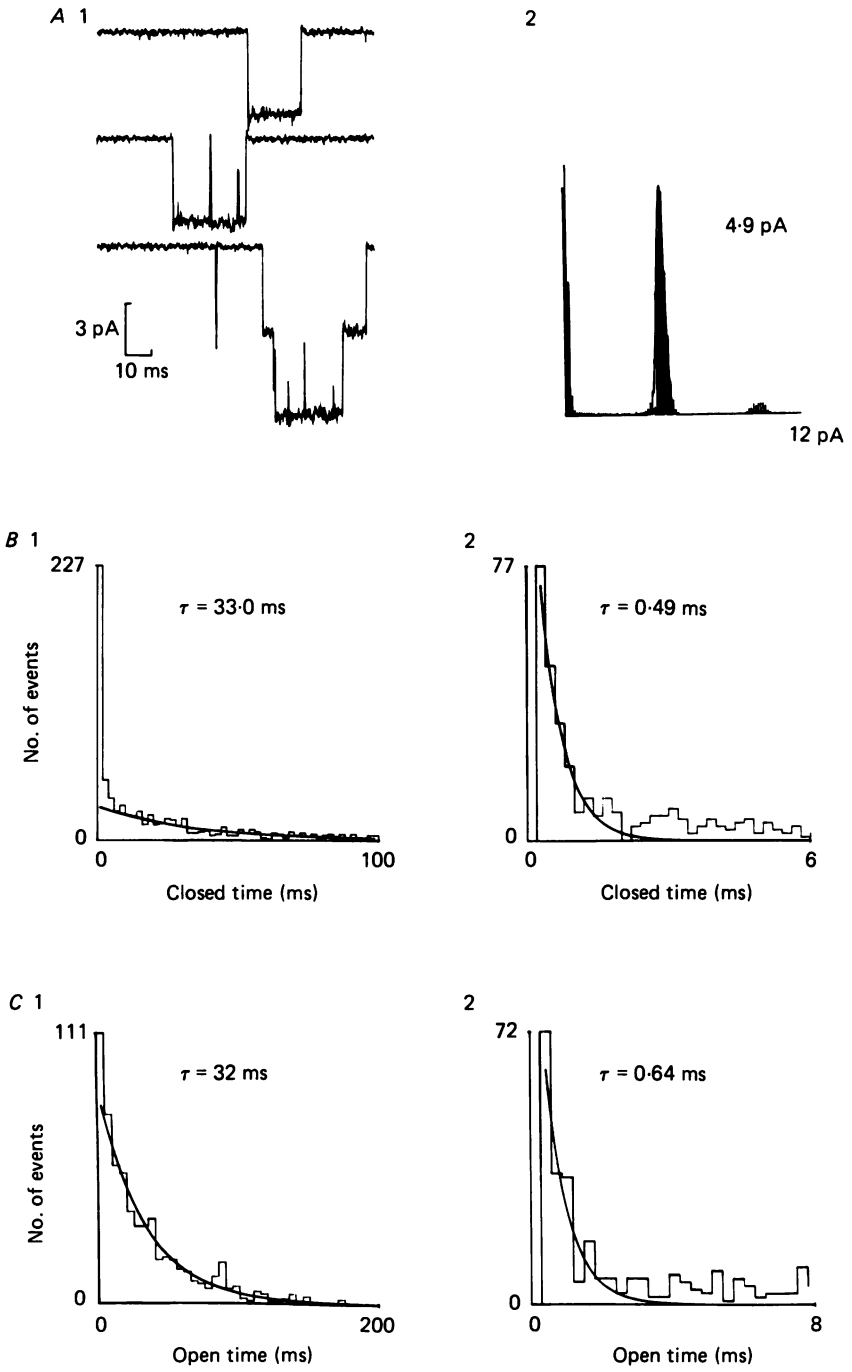

Fig. 1. For legend see opposite.

Fig. 1*B*2, reflects the transient closures (τ = 0·49 ms) of open channels. We chose to exclude transient closures from our estimation of burst times (see Methods) and thus ignored closures shorter than 3 ms, a value 5–6 times longer than the τ of brief closures but much different from the mean time (τ = 33 ms) between openings.

The open duration histogram presented to the left of Fig. 1*C* indicates that most openings were exponentially distributed with a mean burst time of 32 ms in this example. For seven control patches held at −150 to −160 patch potentials, the averaged mean burst time was 32±2 ms (mean ±S.D.). This histogram also shows that there was an excess of brief events and this fast component is presented in detail in Fig. 1*C*2. The mean burst time for the rapid channels was τ = 0·64 ms. In seven control patches, the mean burst time of the rapid channels averaged at 0·64±0·23 ms (mean ±S.D.). In these seven control patches, the rapid channels contributed to only 1–2% of the total open time.

Effects of CPZ and PCP on single ACh-activated channels

When included in the ACh pipette solution, neither CPZ nor PCP affected the single-channel conductance (Fig. 2). For three control patches, the single-channel conductance was 34±4 pS (mean ±S.D.). For four patches exposed to both ACh and

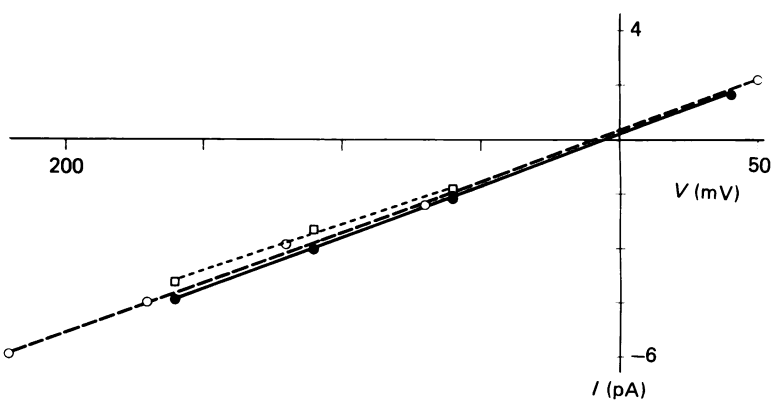

Fig. 2. Effects of CPZ and PCP on single ACh-activated conductance. Current–voltage (*I–V*) curves from three cell-attached patches. ○, control, 200 nM-ACh pipette Ringer solution. ●, CPZ, 100 nM-CPZ included in the 200 nM-ACh pipette Ringer solution. □, PCP, 100 nM-PCP included in the 200 nM-ACh pipette Ringer solution.

CPZ, the slope conductance was 34±3 pS (mean ±S.D.). When the pipette solution contained both ACh and PCP, the single-channel conductance was 35±1 pS (n = four patches; mean ±S.D.). This finding indicates that neither drug induced a fast unresolved flickering or a partial blockade of the ACh-activated channel. Furthermore, when either CPZ or PCP (100–500 nM) was included in an ACh-free pipette solution no ACh-activated channel activity was observed. Thus, neither drug activates the channel, although both CPZ and PCP show, in addition to their non-competitive blocking action a significant interaction with the ACh-binding site (Heidmann *et al.* 1983).

Fig. 3 presents examples of single ACh-activated currents recorded from three

patches exposed to 200 nM-ACh in *A*, or 200 nM-ACh and 100 nM-CPZ in *B* or 200 nM-ACh and 100 nM-PCP in *C*. All three patches were held at a potential 100 mV hyperpolarized from rest (−170 mV transmembrane potential). As indicated above, the single-channel current amplitudes were similar in the three recordings.

In the presence of 100 nM-CPZ (Fig. 3*B*) or 100 nM-PCP (Fig. 3*C*), the burst times appeared shortened. Histogram analysis indicates that for these recordings, the control mean burst time (Fig. 3*A*1) was 30 ms, while in the presence of CPZ or PCP, the mean burst times were reduced to 20 ms or 16 ms, respectively. (For four patches exposed to 100 nM-CPZ, the average mean burst time was 21 ± 2 ms (mean ± S.D.); for four patches in the presence of 100 nM-PCP the average mean burst time was 15 ± 3 ms (mean ± S.D.).

Separate analyses indicated that CPZ and PCP did not affect the fast component. For seven control patches, $\tau = 0{\cdot}4 \pm 0{\cdot}1$ ms (mean ± S.D.). When CPZ was also included in the ACh-pipette solution, the averaged mean open time was 0·5 ± 0·1 ms (n = four patches; mean ± S.D.). Addition of PCP to the ACh-pipette solution resulted in averaged mean burst times of 0·6 ± 0·3 ms (n = four patches; mean ± S.D.). The percentage of open time due to rapid channels was always less than 2%, whether or not CPZ or PCP was present. These values may have errors associated with them due to the resolution (200 μs) of our recordings.

CPZ and PCP effects on single-channel kinetics were further investigated to determine whether these drugs act by blocking open ACh-activated channels (Adams, 1976, 1977; Neher & Steinbach, 1978).

Number of transient closures per burst

As previously mentioned, we observed infrequently transient closures of open channels. For seven control patches, the number of brief closures per burst was 0·6 ± 0·2 (mean ± S.D.) with a mean duration of 0·5 ± 0·1 (mean ± S.D.).

When either CPZ or PCP was included in the ACh pipette solution the number of brief closures per burst was not different from control. For four patches exposed to CPZ, there were 0·8 ± 0·4 (mean ± S.D.) transient closures of 0·5 ± 0·1 mean duration (mean ± S.D.) per burst, and for four patches in the presence of PCP there were 0·5 ± 0·4 transient closures of 0·6 ± 0·3 mean duration per burst (mean ± S.D.). Thus, neither compound elicited rapid transitions between conducting and non-conducting states of the channel. Furthermore, the mean closed times of the brief closures were unchanged, suggesting that no new non-conducting channel state was induced.

Voltage dependence

CPZ and PCP have pK_as of 9·2 and 9·3, respectively, and thus at the experimental pH of 7·2, 99% of the drug molecules are positively charged. The transmembrane potential might influence the actions of these positively charged drugs, if they were to interact with sites within the open channel.

For a given patch, mean burst durations were determined at three different recording potentials: rest, 50 mV hyperpolarized and 100 mV hyperpolarized. Ratios of (1) the mean burst time at 100 mV hyperpolarized potentials as compared to the mean burst time at rest, and (2) the mean burst time at 50 mV hyperpolarized potentials as compared to the mean burst time at rest, were then calculated. The same

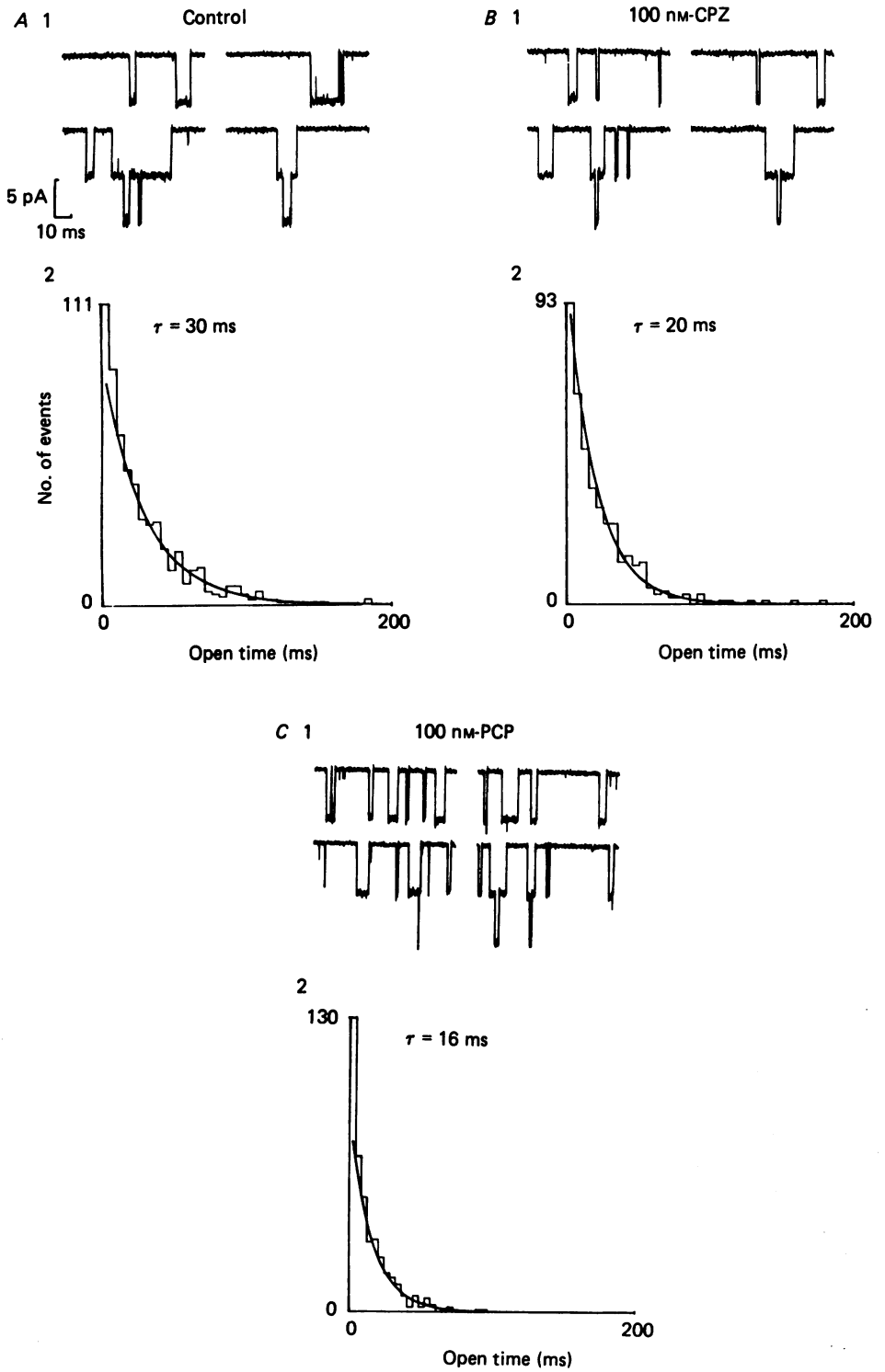

Fig. 3. For legend see opposite.

measurement and their analysis were performed on several control as well as CPZ- or PCP-exposed patches. The results are summarized in Table 1.

For six control patches, a 100 mV hyperpolarization prolonged burst durations by a factor of $2{\cdot}5 \pm 0{\cdot}5$ (mean $\pm$ S.D.). Similarly, in the presence of CPZ mean burst durations were $2{\cdot}4 \pm 0{\cdot}4$ (n = six patches; mean $\pm$ S.D.) times longer at 100 mV hyperpolarized potentials. In the case of PCP, a 100 mV hyperpolarization led to mean burst times that were prolonged $2{\cdot}2 \pm 0{\cdot}7$ (n = four patches, mean $\pm$ S.D.) fold. Membrane hyperpolarization increased mean burst durations to the same extent in the absence as in the presence of CPZ or PCP. The same conclusion was reached when histograms were constructed without excluding brief closures. Thus, CPZ and PCP effects on the mean burst time are surprisingly voltage independent.

TABLE 1. Voltage dependence of CPZ and PCP effect

Patch polarizations ratios	$\tau_{50\ mV}/\tau_{rest}$	$\tau_{100\ mV}/\tau_{rest}$
Control	$1{\cdot}4 \pm 0{\cdot}3$ ($n = 6$)	$2{\cdot}5 \pm 0{\cdot}5$ ($n = 6$)
50 nM-CPZ	$1{\cdot}7 \pm 0{\cdot}1$ ($n = 6$)	$2{\cdot}4 \pm 0{\cdot}6$ ($n = 4$)
50 nM-PCP	$1{\cdot}3 \pm 0{\cdot}1$ ($n = 4$)	$2{\cdot}2 \pm 0{\cdot}7$ ($n = 5$)

Mean $\pm$S.D. $\tau_{50\,mV}$ and $\tau_{100\,mV}$ are the mean burst times at 50 and 100 mV hyperpolarized potentials. τ_{rest} is the mean burst time at rest.

Concentration dependence

A simple sequential channel blocking model predicts that the inverse of the open time (i.e. the sum of the normal channel closure rate and its blocking rate by the effector) will be a linear function of blocker concentration (Adams, 1976, 1977; Neher & Steinbach, 1978). We determined burst durations of ACh-activated channels from patches exposed to concentrations of CPZ or PCP ranging between 10 and 500 nM. At effector concentrations higher than 500 nM, patches tended to be very unstable. Furthermore, as discussed below, at higher effector concentrations channel activity was much reduced.

Fig. 4 shows the inverse of the mean burst duration plotted as a function of effector concentration. For concentration of CPZ ranging between 10 and 200 nM, burst times first became shorter as the concentration increased. A similar trend for PCP was evident in the concentration range of 10–100 nM. At higher concentrations, this type of effect was no longer observed. In fact, the reverse trend took place, the burst time increased at effector concentrations of 200 nM or more. Although the

Fig. 3. Effects of CPZ and PCP on single ACh-activated channels. *A*, control, 200 nM-ACh pipette Ringer solution. Transmembrane potential, −170 mV. 1, single-channel currents recorded from a cell-attached C2 patch in the presence of 200 nM-ACh. 2, open time distribution for the recording for the events presented in 1. The time constant (τ) is 30 ms. *B*, CPZ, 100 nM-CPZ included in the 200 nM-ACh pipette Ringer solution. Transmembrane potential, −170 mV. 1, single-channel currents from another C2 patch in the combined presence of 100 nM-CPZ and 200 nM-ACh. 2, open time distribution for the recording shown in 1. The time constant is 20 ms. *C*, PCP, 100 nM-PCP included in the 200 nM-ACh pipette Ringer solution. Transmembrane potential, −170 mV. 1, single-channel currents from a third C2 patch exposed to 100 nM-PCP and 200 nM-ACh. 2, open time distribution for this recording. The time constant is 16 ms.

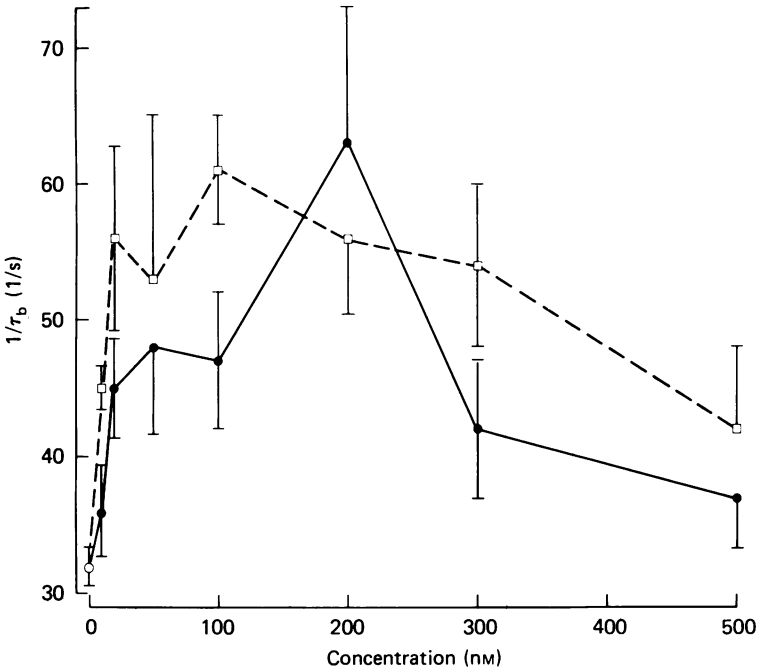

Fig. 4. Effect of CPZ and PCP concentration on the rate of channel closing. ○, control, 200 nM-ACh pipette Ringer solution only; ●, CPZ included in the 200 nM-ACh pipette solution; □, PCP included in the 200 nM-ACh pipette solution. For all patches, the transmembrane potential was -160 ± 10 mV. Each point is the mean for three to eight recordings and has a S.D. bar.

interpretation of these data is not obvious, it is clear that such a concentration profile is not solely accounted for by a single sequential 'channel block' scheme.

Effects of CPZ and PCP on channel activity

The frequency of channel openings ranged from 0·7 to 32 openings/s and the frequency distribution was bimodal. 92 % of the patches had opening frequencies that were less than 13 openings/s and were under one peak of the distribution. The 8 % of patches with high opening frequencies (13–32 openings/s) may have included high density clusters of AChRs and we excluded these patches from the analyses presented below.

Under control conditions (pipette solution containing 200 nM-ACh), the average frequency of channel opening was 5 ± 3 (n = seven patches; mean $\pm$S.D.) with a range of 1·9 to 11 openings/s. This range probably reflects differences in the areas of the membrane patches and in AChR densities. Thus, under control conditions, due to the type of patch configuration used (cell attached), there was considerable variation in the observed opening frequencies. With this reservation in mind, we examined the effect of CPZ and PCP on the opening frequency. The results are presented in Fig. 5. It is clear that in the range of 100–200 nM, neither CPZ nor PCP led to a reduction in the opening frequency. In fact, opening frequencies averaged at higher levels than control, although the differences were not statistically significant. At higher effector

concentration (300–500 nM) opening frequencies were depressed. For example, between 10 and 20 nM-PCP openings occurred at 7·5 ± 3·6/s (n = seven patches: mean ± S.D.), whereas between 300 and 500 nM openings frequencies averged at 3·1 ± 1·2/s (n = seven patches; mean ± S.D.; confidence level > 99·5 %). In the case of CPZ, at concentrations between 10 and 20 nM the opening frequency was 9·6 ± 6·3/s (n = six patches; mean ± S.D.), while at 300–500 nM-CPZ, openings occurred 3·5 ± 2·5/s (n = six patches; mean ± S.D.; confidence level > 99 %). Attempts were made to

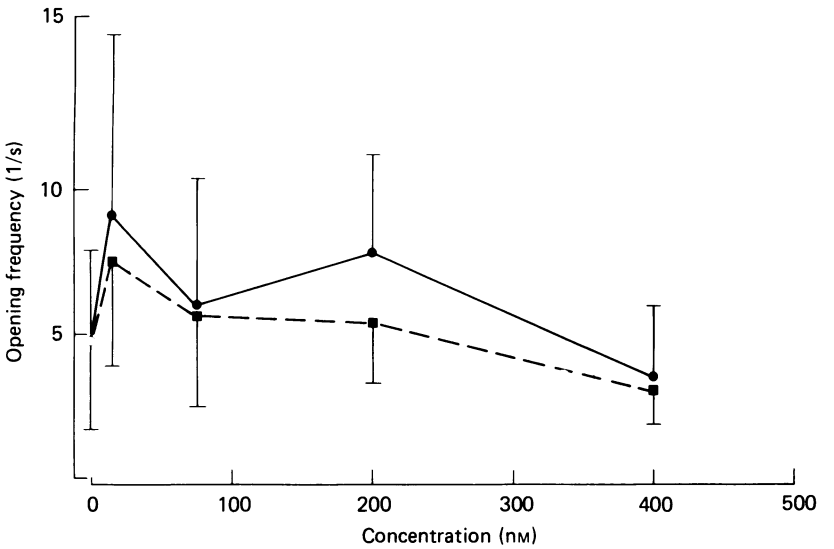

Fig. 5. Effect of CPZ and PCP concentration on channel activity. ○, control, 200 nM-ACh pipette Ringer solution only; ●, CPZ included in the 200 nM-ACh pipette solution; ■, PCP included in the 200 nM-pipette solution. Each point is the mean for four to eight recordings; the bar is the S.D.

pursue this analysis by using outside-out patches and comparing opening frequencies from a single patch before and after effector exposure. However, we were not successful in obtaining stable patches in this configuration. Furthermore, Sine & Steinbach (1984) found that for outside-out patches of BC3H1 cells, there is a time-dependent decrease in channel opening frequency. Such a process would complicate studies of blockers' effects on opening frequency. None the less, our studies indicate that high (300 nM) concentrations of CPZ or PCP led to a reduction in the frequency of channel opening.

Trifluoperazine has effects similar to those of CPZ

Clapham & Neher (1984) observed that 100 nM–1 μM-trifluoperazine (TFP, a phenothiazine derivative related to CPZ) substantially reduced opening frequencies of ACh-activated channels in whole-cell clamped bovine chromaffin cells. At these concentrations, open durations were only slightly reduced. However, in C2 myotubes, under our experimental conditions, the inclusion of 100 nM-TFP in the pipette solution led to a reduction in mean burst times (20 ± 2 ms; n = four patches; mean ± S.D.) without a significant depression in the opening frequency (5·1 ± openings/s).

On the other hand, under the same conditions the non-competitive blocker QX 222 elicited the typical flickering described by Neher & Steinbach (1978) with denervated frog muscle cells (data not shown). A significant difference thus exists between chromaffin cells and skeletal muscle receptors, at least as far as TFP is concerned.

Clapham & Neher (1984) suggested that intracellular actions of TFP contributed to inhibition of exocytosis in chromaffin cells. One could thus wonder if in our experiments, CPZ could also have an intracellular effect. Such an effect would be unlikely since only a patch of membrane of a few square micrometres was exposed to the drug. Nevertheless, we checked this point by adding CPZ to the bath subsequent to seal formation. Even 30 min after CPZ addition, no change in mean burst time occurred. Thus, we do not feel that CPZ effects are due to intracellular actions.

DISCUSSION

Radiolabelled CPZ and PCP have been extensively used *in vitro* to monitor the conformational transitions of the AChR from *Torpedo* (review in Oswald *et al.* 1983; Heidmann & Changeux, 1984; Changeux *et al.* 1984) mostly at the level of a unique high-affinity site sensitive to histrionicotoxin (Eldefrawi, Eldefrawi, Aronstam, Maleque, Wornick & Albuquerque, 1980). In particular, rapid-mixing photolabelling experiments (Heidmann & Changeux, 1984) disclosed a striking enhancement, by several orders of magnitude of the rate of [^{3}H]CPZ incorporation into the AChR, which depends upon CPZ concentration, is agonist specific and disappears with the fast and slow interconversion of the receptor molecule toward high-affinity desensitized conformations (Heidmann & Changeux, 1984 and unpublished observations). The data were interpreted in terms of the selective labelling of a transient conformation which closely parallels, or is even identical, to a physiologically 'active state' where the channel is open. Consistent with these results is the major finding of this study that CPZ and PCP at nanomolar concentrations, reduced, by as much as 50 %, mean burst times of the single channels opened by ACh in cell-attached patches of C2 mouse myotubes. On the other hand, at concentrations higher than 200 nM, CPZ and PCP lead to the opposite effect: a shift back to the long mean burst times found at low concentrations of effector. These two classes of effects will be successively discussed.

The reduction of mean burst times caused by CPZ and PCP looks unconventional. The classical interpretation of such reduction is a steric block of ion transport by a direct interaction with a site present within the open channel. Depending on the range of affinity of the blocking agent for its site, two modes of channel block have been reported (Neher & Steinbach, 1978). The 'fast channel block' occurs for low-affinity blockers in high ranges of concentrations: it manifests itself by a 'flickering' interpreted as due to the binding and unbinding of the blocker at rates which are large compared to the normal rate of channel closure. With the C2 muscle cells such a flickering was observed with the non-competitive blocker QX-222 (data not shown) but *not* with CPZ and PCP in agreement with the rather low concentration range and high affinities at which the latter compounds exert their effect. The 'slow channel block' is found with high-affinity blockers and corresponds to a reduction of the mean burst time, the dissociation of the blocker from the channel being too

slow to produce flickering. Even though CPZ and PCP reduce mean burst times, such a mechanism does not suffice to explain all the effects noticed with these two non-competitive blockers.

First, the ion channel blocked by such high-affinity compounds would not be activated and within a given concentration range opening frequencies might eventually be reduced. However, concentrations (10–100 nM) of CPZ or PCP which reduce open times do not significantly depress channel opening frequencies (Fig. 5). Secondly, the inverse of the burst duration, $1/\tau_b$, should be a linear function of effector concentration, but we did not observe this type of relationship between CPZ or PCP concentration and $1/\tau_b$ (Fig. 4). Finally, one would expect the block of open channels by positively charged compounds to be sensitive to membrane voltage. However, we found that the CPZ or PCP effect of τ_b was voltage independent, even though 99% of the molecules were positively charged at the experimental pH. Thus, the conventional mechanism of a high-affinity steric block of the open ACh-activated channel at the level of a site located within the channel does not suffice to account for our data.

Alternative mechanisms have, thus, been envisaged. For instance, would CPZ and PCP induce new conducting but kinetically different states of the ion channel? In the absence of ACh, neither CPZ nor PCP (100–500 nM) was capable of activating the channel, and thus one would have to suppose that the association of CPZ or PCP with the AChR results in kinetically different channel behaviour. If so, one would expect open duration histograms to include at least one additional exponential component when compared to control histograms. We did not observe any extra exponential in the histograms compiled for recordings from effector exposed patches. However, if the closing rates of effector-associated and effector-free channels were not very different, we would have failed to detect such additional components.

Also, would desensitization account for the reduction in mean burst time? If so, one would expect channel opening frequencies to be depressed. At 10–100 nM, CPZ and PCP caused a reduction of mean burst times (Fig. 4) without decreasing opening frequencies (Fig. 5). Thus, it is unlikely that densitization accounts for the reduction in mean burst time produced by CPZ and PCP.

It is difficult to compare our results with previous electrophysiological studies of the effects of CPZ on the nicotinic response to ACh (Koblin & Lester, 1979; Anwyl & Narahashi, 1980), because the latter were carried out using micromolar concentrations of CPZ or under equilibrium conditions. Koblin & Lester (1979) observed that in *Electrophorus electricus* electroplaques 25 μM-CPZ reduced at equilibrium the amplitude of suberyldicholine-activated currents during depolarizing voltage jumps without affecting relaxation rates. They concluded that this effect was not due to competitive inhibition but rather to an effect of CPZ on membrane lipid. Anwyl & Narahashi (1980) found that 1–3 μM-CPZ led to time-dependent inhibition of repetitive iontophoretic ACh potentials recorded from rat muscle. These authors suggested that CPZ blocked the activated AChR and dissociated slowly from this complex. We did not observe any time-dependent effects of CPZ or of PCP (data not shown). That is, mean burst times measured at the beginning of a recording (30 s – 1 min after gigaseal formation) were not different from mean burst times measured from the same patch tens of minutes later. Furthermore, channel opening frequencies did not show a time-dependent decline. Similarly, for micromolar levels

of PCP, Albuquerque *et al.* (1980) observed time- and voltage-dependent reductions in the peak amplitude of end-plate currents.

We have considered other mechanisms to account for such rather 'unconventional' effects of CPZ and PCP in the nanomolar range of concentrations. In the first, CPZ and PCP effects might be viewed as due to interactions with membrane lipids rather than with the AChR itself as suggested by Koblin & Lester (1978). In *Torpedo* membranes, CPZ has a significant affinity for multiple low-affinity sites presumed to be in contact with the lipid bilayer (Heidmann *et al.* 1983) and distinct from the unique high-affinity site. On this basis, however, one would expect the concentration profiles (Fig. 4) to differ from those observed.

Alternatively, CPZ and PCP may reduce the mean burst time when they bind to high-affinity allosteric sites topographically distinct from the ion channel. However, such a model does not simply account for the available structural data on the known high-affinity site for non-competitive blockers in *Torpedo* receptor. The labelling of all five subunits at the level of this unique high-affinity site (Oswald & Changeux, 1981; Heidmann *et al.* 1983) suggests that it is located along the axis of fivefold symmetry which has been assumed (yet without proof) to trace the path of the permeant ions through the receptor molecule (see Changeux *et al.* 1984; Guy, 1984; Finer-Moore & Stroud, 1984).

Assuming that the structural data about *Torpedo* receptor are still valid for C2 mouse receptor, one may consider that the site at which CPZ and PCP reduce the mean burst time is this unique high-affinity site which would belong to the ion translocation device. However, additional assumptions regarding its structure have to be made. For instance, the walls of the ion transport path might belong to a complex structure which would not be restricted to the peptide segments spanning the lipid bilayer. The high-affinity site would not be located within the membrane but at a more superficial position, where it would no longer be sensitive to electric fields. The model accounts for the voltage, independent shortening of mean burst times. Analysis of the transmembrane folding of the AChR molecule by labelling with radioactive non-competitive blockers (Giraudat, Dennis, Heidmann, Chang & Changeux, 1986) or, indirectly, by *in vitro* mutagenesis (Mishina, Kurosaki, Tabimatsu, Morimoto, Noda, Yamamoto, Terao, Lindstrom, Takahashi, Kuno & Numa, 1984) might lead to the demonstration of the correct model among the diverse ones suggested.

The second class of effects takes place at higher (> 300 nM) concentrations where CPZ and PCP give depressed channel opening frequencies (Fig. 5). At these concentrations (> 300 nM) mean burst times increased and approached control levels as if an 'unblocking' of the ion channel by CPZ and PCP were taking place (Fig. 4). Several mechanisms may account for these phenomena. The trend towards longer burst times may reflect the development of an increased affinity of the receptor for ACh, a process associated with desensitization (review Heidmann *et al.* 1983; Oswald *et al.* 1983). It is also possible that the reduced opening frequency results from a competitive inhibition by CPZ or PCP for the ACh binding site in this high range of concentrations. ACh was routinely used at a concentration of 200 nM; ACh has a dissociation constant, K_D, for the ACh-binding site on the resting AChR of approximately 100 μM (Colquhoun & Sakmann, 1983); CPZ and PCP have K_D of 24 and 250 μM, respectively for the ACh binding site on *Torpedo* membranes (Heidmann

et al. 1983). Thus, one would expect CPZ and PCP to compete with ACh for the receptor binding site under our experimental conditions. However, if the depression in opening frequency were due solely to competitive inhibition, given the tenfold difference in K_D of CPZ and PCP for *Torpedo* ACh-binding site (see above), one would expect CPZ and PCP to exhibit different concentration profiles. We did not observe the large difference expected (Fig. 5). Competitive inhibition thus most likely does not significantly account for the depression in channel opening frequency. On the other hand, Clapham & Neher (1984) observed that trifluoperazine, 100 nM to 1 μM, accelerated and prolonged desensitization caused by the application of 20 μM-ACh to whole-cell clamped chromaffin cells and such a mechanism might account, here, for the depressing effect of high concentrations of CPZ and PCP on channel opening frequency.

Finally, the most plausible explanation of the return to control levels of the mean burst time noticed at high CPZ and PCP concentrations is that, under such conditions, these blockers interact with multiple low-affinity sites such as those demonstrated by *in vitro* binding experiments (Heidmann *et al.* 1983). As a consequence, the receptor molecule would undergo an allosteric transition such that in its active open channel conformation CPZ or PCP would no longer bind with a high affinity. This may for instance result from a global tilt of quaternary structure elicited at the level of the contact area with the lipid bilayer (Heidmann *et al.* 1983). Binding of the non-competitive blockers on the 'external' surface of the receptor molecule would then indirectly affect the more 'internal' high-affinity site for CPZ and PCP. Such a mechanism can be tested since the two categories of sites postulated at low and high concentrations of non-competitive blockers, might possess different pharmacological specificities.

In conclusion, patch clamp studies on the effect of CPZ and PCP point to original features of ACh-receptor function that create challenging constraints, but also may offer useful clues, for modelling the transmembrane organization of its constitutive polypeptide chains.

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