On the Mechanism of Desensitization in Quisqualate-Type Glutamate Channels

O. TOUR, H. PARNAS, AND I. PARNAS

The Otto Loewi Center for Cellular and Molecular Neurobiology and the Department of Neurobiology, the Hebrew University, Jerusalem 91904, Israel

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Tour, O., H. Parnas, and I. Parnas. On the mechanism of desensitization in quisqualate-type glutamate channels. J Neurophysiol 84: 1–10, 2000. Desensitization of crayfish glutamate channels was studied in outside-out patches employing an improved fast drug-application technique. Low concentrations of glutamate produced substantial desensitization without correlation with the detected number of open channels. The desensitization time constant ($\tau_D$) was found to be independent of glutamate concentration (0.3–20 mM). These results suggest that in addition to desensitization from a state of fully liganded channels, a substantial fraction of desensitization occurs also from channels in a partly-liganded state. A kinetic model was developed. The model accounts for the multifaceted behavior of desensitization as well as for resensitization.

Introduction

Desensitization of glutamate channels is of physiological importance. For example, it may contribute to the shaping of the time course of synaptic currents of the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate (AMPA/KA) glutamate channels of the chick CNS (Trussell and Fischbach 1989; Trussell et al. 1993) and of the quisqualate type glutamate channel of the crayfish neuromuscular junction (NMJ) (Dudel et al. 1990). In contrast, when slower desensitization of AMPA channels was observed (Colquhoun et al. 1992; Hestrin 1992), it was argued that desensitization does not play a role in determining the time course of the synaptic current.

Because of the relatively slow (tens to hundreds of milliseconds) resensitization, desensitization may also limit the frequency at which AMPA receptors can produce full-amplitude responses to glutamate (Jones and Westbrook 1996; Otis et al. 1996). Another physiological effect of desensitization is demonstrated by the finding that a very low concentration of glutamate significantly reduces the availability of activatable channels (Dudel et al. 1990; Heckmann and Dudel 1997; Heckmann et al. 1996; Kiskin et al. 1986; Tour et al. 1995; Trussell and Fischbach 1989), probably due to accumulation of channels in a desensitized state.

Despite considerable progress in understanding various aspects of desensitization (reviewed by Jonas and Spruston 1994; Jones and Westbrook 1996), the receptor states from which desensitization or resensitization occurs need further study for most cases examined. Earlier studies suggested that desensitization occurs from a fully liganded state of the receptor as well as from a partly liganded state (Raman and Trussell 1995; Trussell and Fischbach 1989 for glutamate channel in the chick; Dudel et al. 1990, 1993 for a very fast and completely desensitizing glutamate channel in the crayfish; Tour et al. 1995 for an incompletely desensitizing glutamate channel in the crayfish; Heckmann et al. 1996 for GluR6 expressed in HEK 293 cells; Heckmann and Dudel 1997 for the Drosophila larval glutamate channel).

In the present study, we investigated mechanisms of desensitization and resensitization of glutamate channels taken from crayfish muscles. Desensitization and resensitization were studied under a variety of experimental protocols and in response to a wide range of glutamate concentrations. To do so, we further improved the fast drug-application technique (Dudel et al. 1990; Franke et al. 1987; Tour et al. 1995). Our improved technique reduced the variability of the raw data and enabled us to design complex experimental protocols in which the patch of membrane is exposed to three different solutions that can be switched at intervals in the sub-millisecond range.

With this technique, we demonstrate that desensitization occurs both from the fully liganded and the partly liganded states. The latter predominates at low concentrations of glutamate and occurs without detectable prior channel opening.

Methods

Preparation and solutions

Deep abdominal extensor muscles (Parnas and Atwood 1966) were isolated from crayfish (Procambarus clarki) 4–8 cm in length. The isolated muscles were bathed in standard Van Harreveld solution containing (in mM) 220 NaCl, 5.4 KCl, 13.5 CaCl$_2$, 2.5 MgCl$_2$, and 10 tris-maleate buffer. The pH was adjusted to 7.4 by addition of NaOH. The bath temperature was controlled at 8–10°C. Patch electrodes were filled with low Cl$^-$ intracellular solution (Franke et al. 1986). The solution contained (in mM) 150 K-propionate, 5 Na-propionate, 10 MgCl$_2$, and 10 EGTA to establish a free Ca$^{2+}$ concentration of $10^{-8}$ M and 10 mM Tris-maleate buffer with the pH adjusted to 7.2 by the addition of KOH. To obtain G1 seal, we treated the muscles with 0.3 mg/ml collagenase (Sigma; type Ia) for 20 min at 20–22°C.

Fast application technique

In a previous study (Tour et al. 1995), the “double-ticker” (a modification of the fast application system “ticker”) (Dudel et al. 1990) was employed. The model accounts for the multifaceted behavior of desensitization as well as for resensitization.
that the fast application systems work properly.

For each outside-out patch studied, we first ascertained that the three objects—two fast application tubes and the patch electrode tip—were properly positioned. We did this by checking for similarity of the response to 10 mM glutamate applied by the two tickers. The two congruent currents shown in Fig. 1B indicate a proper glutamate application. Such a control was conducted on each patch tested.

While glutamate application was rapid, the washing out of glutamate was relatively slow (−3 ms; not shown). This slow washing may distort results of twin pulse experiments designed to measure the time course of resensitization. This, however, was not the case, since resensitization is much slower (50% resensitization is reached following −120 ms; see Fig. 5B).

Outside-out patches and recording

Patch-clamp recording (Axopatch-1D, Axon Instruments, Foster City, CA) was performed in the outside-out configuration (Hamill et al. 1981). Thin-wall borosilicate capillaries with an inner filament (GC150TF-10, CEI, England) were pulled in two stages using the computerized DMZ horizontal pipette puller (Augsburg, Germany). Pipette resistance was 2–7 MΩ. Normally, 1–20 glutamate channels were present in a patch. The holding potential was −70 mV.

Current traces were filtered (2 or 10 kHz; Axopatch-1D), digitized at 20 or 100 kHz (DigiData 2000 interface, Axon Instruments), and fed on-line to a computer. Data was analyzed with the pCLAMP6 software (Axon Instruments).

Run-down was accounted for by alternating many times between the test glutamate pulse and the control glutamate pulse. By this procedure, the data from the two pulses were collected over the same period of time. Thus run-down effects were the same for the two pulses (for details, see Tour et al. 1995). Data are given as means ± SD.

Computer simulations

Computer simulations were performed on a Silicon Graphics (Indy) computer using the BIOQ software developed in our laboratory for modeling chemical and biochemical reactions (more information on BIOQ can be found at http://www.ls.huji.ac.il/~parnas/bioq/bioq.html).

Results

Characterization of the desensitization process

Figure 1A demonstrates desensitization of glutamate channels in response to a pulse of 10 mM glutamate given for 460 ms. The ensemble current reached its peak ($I_{\text{peak}}$) within 400 µs and then declined to a steady-state response ($I_{ss}$).

The decay of the current could be fitted by a single exponential with a time constant ($\tau_D$) of 19.3 ms and the ratio $I_{ss}/I_{\text{peak}}$ was 0.21 (the average $\tau_D$ was 16.3 ± 6.3 ms and the ratio $I_{ss}/I_{\text{peak}}$ was 0.33 ± 0.17; $n = 8$). As the average value of $\tau_D$ is 16.3 ms, it follows that much shorter applications of glutamate may be employed. To check whether indeed shorter applications (~5 times that of $\tau_D$) will yield the correct value of the ratio $I_{ss}/I_{\text{peak}}$, we fitted an exponential to the initial 80 ms of the current of Fig. 1A and extrapolated the fit to the rest of the current. As seen, the extrapolation coincides with the long current. In 49 experiments, with a 80-ms duration of application, the average $\tau_D$ was 17.6 ± 5.9 ms and the average $I_{ss}/I_{\text{peak}}$ ratio, evaluated from the constant (C) of the exponential function, was 0.31 ± 0.16.

Dependence of $\tau_D$ on glutamate concentration

Franke et al. (1993) and Buchman and Parnas (1999) showed that when desensitization occurs primarily from the doubly liganded state (as for the nicotinic receptor), $\tau_D$ declines monotonically as agonist concentration rises. On the other hand, when desensitization occurs also from other states of the receptor, $\tau_D$ exhibits a complex dependence on agonist concentration.

Usually, there is a large variance in $\tau_D$ (see RESULTS above) (see also Franke et al. 1993; Heckmann and Dudel 1997). Such a variation may distort the true dependence of $\tau_D$ on agonist concentration. To overcome this difficulty, the dependence of $\tau_D$ on glutamate concentration was measured using the double-ticker (Tour et al. 1995). The double-ticker enables a concomitant rapid exchange among three solutions: a reference concentration (10 mM), a test concentration, and a glutamate free solution (for washing; see for example Fig. 2). Each of the $\tau_D$'s
obtained for the test concentration is then normalized to the $\tau_D$ obtained concurrently at the reference concentration (10 mM) from the same patch.

Figure 2A depicts an example in which the test concentration was 0.5 mM. The corresponding protocol is shown above the current traces: here,ticker-1 applied the test concentration and ticker-2 applied the reference concentration. Each application lasted 100 ms and was preceded and followed by 700 ms of washing with glutamate-free solution (sufficient time for complete resensitization of the channels) (Dudel et al. 1990; and see resensiti-
zation results in the following text). Three traces of single channel recordings in response to 0.5 (left) and 10 mM (right) glutamate are shown. Such glutamate pulses were repeated 120 times to collect sufficient data for averaging. The average currents are plotted in Fig. 2A, bottom. The decay phases of the currents were fitted by a an exponent (Fig. 2B, - - -), with a $t_D$ of 16.4 ms for 10 mM glutamate and 15.9 ms for 0.5 mM glutamate. The similar values of $t_D$ at the two remote concentrations already suggest, as will be further corroborated, that the glutamate concentration has almost no effect on $t_D$.

The dependence of $t_D$ on glutamate concentrations is summarized in Fig. 2C. It is apparent that $t_D$ is nearly constant for a wide range of glutamate concentrations (0.3–20 mM).

**Predesensitization by a low concentration of glutamate**

The independence of $t_D$ on glutamate concentration suggests that desensitization occurs from at least two states of the receptor, the fully liganded state and a partly liganded state (Buchman and Parnas 1999; Franke et al. 1993).

To further corroborate this conclusion, the response to a given (test pulse) concentration of glutamate was measured without and following preexposure of the patch to a low concentration of glutamate, insufficient by itself to cause a significant channel opening (Colquhoun et al. 1992; Dudel et al. 1990; Heckmann and Dudel 1997; Heckmann et al. 1996; Kiskin et al. 1986; Tour et al. 1995; Trussell and Fischbach 1989).

The results of such predesensitization experiments are shown in Figs. 3 and 4 for a wide range of low glutamate concentrations. In Fig. 3A, each elementary protocol (shown at the top) included two 40-ms test pulses of 2.5 mM glutamate applied by ticker-1. The 1st test pulse (TC) was preceded by 700 ms of exposure to glutamate-free solution (wash), while the 2nd test pulse (TPL) was preceded by application of glutamate-free solution for 590 ms, followed by 110-ms preapplication (denoted PA) of 0.03 mM glutamate ejected from ticker-2 (like TC, TPL is preceded with 700 ms of low glutamate or no glutamate at all). Two examples of raw data are shown: top example has one short opening (*) during PA, and bottom trace is an example of PA in which no channel opening was observed. B: 5 additional PAs from this patch at a higher resolution. Only the 2nd and the 4th traces contain channel openings. C: ensemble currents (average of 61 repetitions of the elementary protocol on the patch shown in A) of TC and TPL are depicted superimposed. The peak amplitude is the sum of 2 phases: $I_o$ (derived from the constant C of a fit function, not shown), and the desensitized phase, denoted as $I_D$. The two vertical bars (right) indicate these two phases for TC.
lower $I_{\text{peak}}(\text{TPL})$. It is possible that openings that occurred during the preapplication (PA) period were followed by desensitization from the doubly liganded state, and this desensitization was the reason for the lower $I_{\text{peak}}(\text{TPL})$. To examine this possibility, the raw data of each PA were inspected for channel openings.

Figure 3B depicts 5 PAs from this patch at a higher resolution. Special measures were taken to increase our ability to detect possible short openings. In particular, the temperature was kept at 10°C, the low-pass filter was 10 kHz, and the sampling frequency was 100 kHz.

In Fig. 3B, only the second and the fourth traces contain channel openings. We searched for channel openings in all 61 PA repetitions applied to this patch. The distribution was: 22 zeroes, 26 single openings, 9 double openings, and 4 triple openings—a total of 56 observed openings. TC and TPL of the 61 episodes were averaged, and the resulting average currents are displayed together in Fig. 3C. A comparison of the average TC with the average TPL reveals that the PA decreased the peak amplitude of TPL relative to TC by 24 pA. At a holding potential of $\sim$70 mV, the single channel current ($i$) is 6 pA. This implies that, on average, 4 fewer channels were opened per episode in $I_{\text{peak}}(\text{TPL})$; in the 61 episodes, a total of 244 fewer channels were opened in $I_{\text{peak}}(\text{TPL})$. Clearly, the 56 openings observed in PA could not have caused desensitization from the doubly liganded state of 244 channels.

The lack of correlation between openings occurring during the PA, and the degree of reduction in $I_{\text{peak}}(\text{TPL})$ shown in Fig. 3, supports the conclusion reached previously (from the data depicted in Fig. 2) that desensitization occurs also from a partly liganded state. Figure 3C shows that $I_{\text{ss}}$ is not affected by desensitization; hence, only a fraction of $I_{\text{peak}}$ (denoted $I_{\text{ss}}$) is reduced by desensitization [see Fig. 3C for notation and also notice that the average ratio $I_{\text{ss}}(\text{TPL})/I_{\text{ss}}(\text{TC})$ was found to be 0.97 ± 0.11 in other 31 patches]. Therefore it is the ratio $I_{\text{ss}}(\text{TPL})/I_{\text{ss}}(\text{TC})$ that quantifies the magnitude of desensitization.

Figure 4 summarizes the degree of desensitization caused by five low concentrations of glutamate (0.01, 0.03, 0.05, 0.1, and 0.3 mM). Clearly, increasing the glutamate concentration during the PA decreased the proportion $I_{\text{ss}}(\text{TPL})/I_{\text{ss}}(\text{TC})$.

Characterization of the resensitization process

We determined the time course of resensitization (recovery from desensitization) by delivering two glutamate pulses—a conditioning pulse (CP) and a test pulse (TP)—and varying the interval between them. The results presented in the preceding text suggest that desensitization occurs from at least two states of the receptor, a doubly liganded state and a partly liganded state. Therefore the resensitization experiments were conducted under two conditions. Under one condition, trials were conducted with a low concentration CP (0.05 mM glutamate) expected to cause significant desensitization from the partly liganded state. Under the second condition, trials were performed with a high concentration CP (2.5 mM glutamate), which is expected to cause desensitization primarily from the doubly liganded state.

The results of these experiments are shown in Fig. 5. Figure 5A, top, depicts results obtained without CP and serves as a control. In Fig. 5A, middle and bottom, 0.05 mM glutamate was applied during CP (the inter-pulse interval was 140 ms for the middle and 0 for bottom). $I_{\text{peak}}(\text{TP})$ was lowest with the zero inter-pulse interval. $I_{\text{peak}}(\text{TP})$ increased with the 140-ms interval but was still smaller than in the control.

In Fig. 5B, the fraction of resensitization is plotted as a function of the inter-pulse interval for 0.05 (●) and 2.5 mM (○) conditioning pulses. In both cases, 50% resensitization is reached at an interval of $\sim$120 ms. This suggests that the rate limiting step for resensitization is common to both routes of desensitization.

Kinetic model

Based on the cumulative experimental results presented here and by Tour et al. (1998), we suggest (Fig. 6D) a kinetic scheme for activation, desensitization, and resensitization of the crayfish glutamate receptor. Figure 6, A–C, shows the steps by which we developed this model; each step is based on the experiments most relevant to that step.

GLUTAMATE CHANNEL ACTIVATION. The kinetic scheme in Fig. 6A describes glutamate channel activation. R stands for free receptor, GR and G2R stand for the single- and double-ligated receptor, respectively, and G2O denotes the open channel. The values of the rate constant for channel opening ($\beta$) and for channel closing ($\alpha$) were determined from single-channel measurements (Tour et al. 1998) at low glutamate concentration. We derived $k_{-2}$ from the time constant of the primary burst (Colquhoun and Hawkes 1981), and we assumed $k_{-1}$ to be equal to $k_{-2}$. With these rate constants determined, $k_1$ and $k_2$ were adjusted to fit the experimental $I_{\text{peak}}$ dose-response curve (see Fig. 8A). The dose-response curve showed an initial slope of 1.7, indicating two binding sites. We also checked for $I_{\text{ss}}$ dose-response curve and found that there is a difference between the $K_d$'s (glutamate concentration that provides half-maximal response) of the two dose response curves: the $I_{\text{ss}}$ dose-response curve has a lower $K_d$ ($K_{d,\text{ss}} = 0.54$ mM) than the $I_{\text{peak}}$ ($K_{d,\text{peak}} = 0.72$ mM) dose-response curve (see experimental results in Fig. 8A, inset).

ESTIMATION OF $K_{d,\text{ss}}$ AND $K_{d,\text{peak}}$. To account for the observed desensitization at high glutamate concentrations (Fig. 1), a desensitized state G2D must be linked to a fully liganded state: i.e., G2R or G2O. As the two options result in an identical
behavior, we decided to link \( G_2 \) to \( G_2 O \) (Fig. 6B). The corresponding desensitization rate constant, \( k_{D2} \), was taken to be the reciprocal of the experimentally observed average \( \tau_D \) at high glutamate concentration (10 mM). The backward rate constant, \( k_{-D2} \), was naturally fixed to account for the ratio \( I_{ss}/I_{peak} \) observed experimentally at this concentration.

The model of Fig. 6B disagrees with two aspects of the experimental results. Contrary to experiments (see Fig. 2C), it predicts that \( \tau_D \) will decline as glutamate concentration increases. Furthermore, it generates a \( K_D \) lower (0.27 mM) than found experimentally (0.54 mM). These discrepancies can be reconciled if another desensitized state is added.

As desensitization from \( G-R \) does not alter the behavior of the model in Fig. 6B, the additional desensitization state must be linked to either \( R \) or \( GR \). Desensitization from \( R \) does not affect the qualitative behavior of model 6B, it only changes the initial concentration of free \( R \). It follows, therefore that only the addition of desensitization from \( GR \) can remove the limitation of model 6B, and hence, model 6C is suggested.

**ESTIMATION OF \( K_D 1 \) AND \( K_2 D1 \).** With desensitization from \( GR \) added, the values of \( k_{D2} \) and \( k_{-D2} \) were re-determined. The values of the two desensitization rate constants (\( k_{D1} \) and \( k_{D2} \)) and the two backward rate constants (\( k_{-D1} \) and \( k_{-D2} \)) were set

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**FIG. 5.** Resensitization from low and high glutamate concentrations (0.05 and 2.5 mM) applied during the conditioning pulse (CP: 92 ms). The test pulse (TP: 100 ms) concentration was 2.5 mM. The experimental protocol was repeated at 0.5 Hz. A: example of 3 ensemble currents (60 repetitions) from 1 such experiment. Top: example is without CP and serves as a control. Middle and bottom: 0.05 mM glutamate was applied during CP with an inter-pulse interval of 140 and 0 ms, respectively. B: the proportion of resensitization [expressed as the ratio between \( I_D(\text{TP}) \) following a conditioning pulse and control \( I_D(\text{TP}) \)] as a function of the inter-pulse interval. Concentrations of the conditioning pulses were 0.05 mM (●, \( n = 7 \)) and 2.5 mM (○, \( n = 6 \)).

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**FIG. 6.** Step-by-step construction of a kinetic model for the channel behavior. A: sequential kinetic scheme describing channel activation. \( R \) is the free receptor channel, \( GR \) and \( G_2 R \) are the receptor with 1 or 2 bound molecules of glutamate, and \( G_2 O \) is the channel in the open state. The channel opening and closing rate constants are \( \beta \) and \( \alpha \), respectively. The glutamate association and dissociation rate constants are \( k_1 \) and \( k_2 \) and \( k_2 \) and \( k_1 \) respectively. B: a desensitized state \( G_2 D \), linked to \( G_2 O \), was added with the desensitization rate constant, \( k_{D2} \), and the backward rate constant, \( k_{-D2} \). C: another desensitization state, \( GD \), was added. GD is linked to \( G_2 D \) and \( G_2 O \). The forward and backward rate constants from \( GD \) to \( G_2 D \) are \( k_3 \) and \( k_{-3} \), respectively.
MECHANISM FOR RESENSITIZATION. The model shown in Fig. 6C predicts that following application of glutamate at a high concentration, late openings will result as the desensitized channel, G₂D, switches to the open state on its way to the unbound state R. Such late openings were never observed experimentally; therefore resensitization must bypass the open state. Thus in Fig. 6D, we added a link between G₂D and GD, with glutamate association and dissociation rate constants $k_4$ and $k_{-4}$. The value of $k_4$ was taken to be equal to $k_1$ because we assumed that agonist association is likely to be independent of receptor state. Similar considerations were made concerning the nicotinic ACh receptor (Franke et al. 1993). $k_{-4}$ was set to obey the rule of microscopic reversibility.

With these values of $k_{-4}$, $k_{-D1}$, and $k_{-1}$, the rate of resensitization predicted by model 6C, is $\sim 40$ ms. The observed rate of resensitization, however, is slower (as shown in the preceding text, 50% resensitization is reached at intervals of $\sim 120$ ms). Therefore an additional slower route for resensitization was added; from GD through D to R. Note that in model 6D an additional desensitized state (D) had to be included.

ESTIMATION OF $k_5$, $k_{-5}$, $k_{D0}$, AND $k_{-D0}$. Glutamate association rate constants to D, and dissociation from GD, are $k_5$ and $k_{-5}$, respectively. The value of $k_5$ was taken to be equal to $k_1$ (see preceding text considerations concerning $k_4$), and $k_{-5}$ was set to an appropriate value to obey the rule of microscopic reversibility. The rate constant of transition from D to R is $k_{-D0}$. Its value was set to be the rate limiting step in the slow resensitization that occurs via G₂D→GD→D→R. The rate constant of transition from R to D is $k_{D0}$. Its value was set to minimize the number of sleeping channels [Dudel et al. (1990) showed that in the case of crayfish glutamate channels, the fraction of sleeping channels is negligible]. The values of the various rate constants are listed in Table 1.

The model of Fig. 6D was developed in steps; for each step, only a few rate constants were considered. Therefore it is important to examine whether the final model with all rate constants included still accounts for our key experiments. We begin by showing examples of simulated ensemble currents (Fig. 7, A and B). The simulations were performed under the same “experimental protocols” as those shown in Figs. 1A and 3C, respectively. Figure 7A depicts simulated (- - -) and experimental (-----) currents obtained in the presence of 10 mM glutamate. The two currents overlap. We chose to depict the current of this particular patch because its $\tau_D$ of 17 ms and its $I_{ss}/I_{peak}$ ratio of 0.3 are similar to the average values obtained from 49 patches.

In Fig. 7B, we show simulations (- - -) of currents without (TC, larger current) and following preapplication (TPL, smaller current) of 0.03 mM glutamate. Experimental results of Fig. 3C are plotted superimposed for comparison. The larger simulated (- - -) and experimental (-----) currents were normalized, each to its own $I_{peak}$.

### TABLE 1. Summary of the rate constants used in simulating the scheme of Fig. 6D

<table>
<thead>
<tr>
<th>Rate Constants</th>
<th>Value</th>
<th>Units</th>
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<tr>
<td>$k_1$</td>
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<td>ms$^{-1}$ mM$^{-1}$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>7</td>
<td>ms$^{-1}$ mM$^{-1}$</td>
</tr>
<tr>
<td>$k_{-1}$</td>
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<td>ms$^{-1}$</td>
</tr>
<tr>
<td>$k_{-2}$</td>
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<td>ms$^{-1}$</td>
</tr>
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<td>$\beta$</td>
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</tr>
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FIG. 7. Examples of the simulations (- - -) of the model of Fig. 6D, superimposed on the relevant experimental results (-----). Polarity of the simulated currents were reversed to conform with the experimental currents. A: ensemble currents resulting from application of 10 mM glutamate. Simulated (- - -) and experimental (-----) currents were normalized, each to its own $I_{peak}$. B: simulations of currents without (TC, larger current) and following preapplication (TPL, smaller current) of 0.03 mM glutamate. Experimental results of Fig. 3C are plotted superimposed for comparison. The larger simulated (- - -) and experimental (-----) currents were normalized, each to its own $I_{peak}$.
differences are expected because the simulations represent an average behavior that may differ from any single experiment.

Figure 8, A–E, compares experimental (data points ± SD without a connecting line) and simulated (—) results of four aspects most relevant in checking the model. Figure 8A shows experimental $I_{\text{peak}}$ (■) and $I_{\text{ss}}$ (○) dose-response curves. All responses were normalized to $I_{\text{peak}}$ at 10 mM glutamate. The model (scheme Fig. 6D), which includes desensitization and resensitization, generates curves that are very similar to the experimental dose-response curve. We found that $K_{dI_{\text{ss}}}$ is smaller than $K_{dI_{\text{peak}}}$. To demonstrate this finding, the two dose-response curves are (Fig. 8A, inset) normalized, each to its own peak at 10 mM glutamate.

The independence of $\tau_D$ on glutamate concentration is shown in Fig. 8B (same results as in Fig. 2C). Here too, simulation results (—) agree well with experimental results. In the simulations shown in Fig. 8B, we calculated $\tau_D$ for a very low glutamate concentration of 0.1 mM and found it to be
larger than in higher concentrations. This concentration is too low to check for $\tau_0$ experimentally. However, it can be seen that the tendency for a larger $\tau_0$ is hinted already in the lowest glutamate concentration measured experimentally (0.3 mM). The simulations also capture this tendency.

Figure 8C shows simulated (---) and experimental (●) results of predesensitization by a low concentration of glutamate. The simulations agree well with the trend seen in the experiments. Furthermore, a quantitative agreement is seen in cases where low concentrations of glutamate were preapplied (0.01, 0.03, and, to a lesser extent, to 0.05 mM glutamate). For the cases of higher preapplied glutamate (0.1 and 0.3 mM), while the simulations agree qualitatively with the experiments, the simulations show weaker predesensitization.

Simulation of desensitization is shown for the two cases of 0.05 mM (Fig. 8D, ---) and 2.5 mM (Fig. 8E, —) glutamate applied during the conditioning pulse (CP). The experimental results at these two conditions are also plotted (● for 0.05 mM in CP; ○ for 2.5 mM in CP; same results as in Fig. 5B). Both simulations fit well with the experimental results except in Fig. 8E, at short intervals.

Evaluating the permitted range of $k_{D1}$

The model parameters were determined on the basis of the relevant experimental results (see preceding text), and hence no sensitivity analysis is required. However, as $k_{D1}$ is the key rate constant controlling desensitization from the single liganded receptor, we examined the permitted range for $k_{D1}$. This was done by multiplying (---) and dividing (-----) $k_{D1}$ by a factor of five and examining the effect on the four kinetic aspects of Fig. 8.

In pursuing this analysis, we recall that in the model of Fig. 6D, $k_{D1}$ is part of two loops. The rule of microscopic reversibility requires that a change in the value of $k_{D1}$ be compensated by an appropriate alteration of another rate constant within each loop. We found that the most suitable parameters to change were $k_{-3}$ and $k_{-4}$, as they have minimal effect on the model’s behavior. Moreover, only these two parameters were not determined on the basis of experimental results, so it is of interest to check the outcome of variation in their values. Figure 8A demonstrates that variations in $k_{D1}$, $k_{-3}$ and $k_{-4}$ virtually do not affect the $I_{\text{peak}}$ dose response, whereas they do affect the $I_{\text{off}}$ dose response: multiplying $k_{D1}$ by a factor of 5 shifts the $I_{\text{off}}$ dose response to the right, causing $K_d I_{\text{peak}}$ to become similar to $K_d I_{\text{peak}}$. Concerning the dependence of $\tau_0$ on glutamate concentration, predesensitization and resensitization (Fig. 8, B–E), multiplying $k_{D1}$ by a factor of 5 affects these kinetic aspects markedly, weakening the agreement between simulation and experimental results. We conclude that the model of Fig. 6D, together with the values of the rate constants provided in Table 1, accounts well for the multifaceted behavior examined in this study.

Discussion

Our results are consistent with the existence of desensitization from a partly liganded state of the crayfish glutamate channel. Using a kinetic model and experiments specifically designed for this purpose, we were able to identify the partly liganded closed state from which desensitization takes place. The findings that the number of binding sites for this channel is two (Fig. 8A) and that $\tau_0$ is independent of glutamate concentration (Fig. 2C) led to the conclusion that it is the single liganded state, GR, in addition to the fully liganded state from which desensitization occurs. Furthermore, the rate constant of desensitization from GR is about half that from $G_2O$.

It is possible that some of the desensitization that is attributed to a single-ligated state in fact reflects desensitization from a doubly liganded state with very short and unresolved openings. If this was the case, we would expect that such short undetectable openings that lead to desensitization will occur also at high glutamate concentrations. This is unlikely to predominate, however, because it would imply low open probability at high glutamate concentrations, and this is not the case (Dudel et al. 1990).

Another aspect that deserves attention concerns the weaker predesensitization predicted by the model at high preapplied glutamate concentrations (see Fig. 8C). The most effective way to increase the simulated predesensitization is to increase $k_{D1}$. The lower line (---) in Fig. 8C is the result of simulating predesensitization with a five times larger $k_{D1}$. Here the agreement between simulation and experimental results at 0.1 and 0.3 mM is better, but multiplying $k_{D1}$ by five worsened the agreement at the low concentrations of glutamate (0.01, 0.03, and 0.05 mM). Multiplying $k_{D1}$ by five also worsens the agreement between simulation and other aspects of the experimental results (see for example Fig. 8B, - - -).

An additional way to increase the simulated predesensitization is to increase $k_1$ (or decrease $k_{-1}$). This, however, shifts the simulated dose response to the left and, hence, will abolish the fit with the experimental results seen in Fig. 8A.

We suggest that resensitization occurs mainly via the transitions $G_3D\rightarrow GD\rightarrow D\rightarrow R$. This conclusion is based on excluding the possibilities of resensitization via other states of the receptor. We reject the possibility of resensitization from $G_2O$ or $G_1R$ because channels did not open after the glutamate was washed from the patch. Resensitization via $G_2D\rightarrow GD\rightarrow GR\rightarrow R$ does exist in the model, but it is much faster than the experimental results due to the high value of $k_{-D1}$. This value was set to produce $I_{\text{off}}$ and $I_{\text{peak}}$ dose-response curves with $K_d$’s matching the experimental results. The difference between these two dose-response curves (Fig. 8A, inset) is determined primarily by the relative values of $k_{-D1}/k_{D1}$ and $k_{-D2}/k_{D2}$.

How robust is the model and to what extent do the conclusions reached depend on the values of the various rate constants? To answer this question, we recall that the model was developed in steps. Each step, including values of rate constants, was directly based on relevant experiments. We then ensured that the complete model accounts for the four most relevant experiments (Fig. 8). When we constructed the model this way — in steps — the only free parameters were $k_{-3}$ and $k_{-4}$. Their values were determined by applying the rule of microscopic reversibility. Nevertheless in view of the key role of $k_{D1}$ in determining the magnitude of desensitization from GR, we conducted a sensitivity analysis of the model, examining the predicted results of the four most relevant aspects (Fig. 8) on changes in the value of $k_{D1}$.

It should be noted that although our model is extremely simple, it reproduces a wide range of experimental results.

We now compare our results to earlier studies. Concerning invertebrate glutamate channels, Heckmann and Dudel (1997)
studied the desensitization and resensitization of glutamate channels in muscles of Drosophila larvae. The proposed reaction scheme requires that five glutamate binding sites be occupied to open the channel. According to this scheme, desensitization occurs from the channel with three and five glutamate molecules attached, and the rate constant of desensitization from the former is much larger.

A similar kinetic scheme to that of Heckmann and Dudel (1997) had been suggested also for the completely desensitized glutamate channel of the crayfish (Dudel et al. 1990, 1993). In particular, five binding sites were assumed and desensitization was taken to occur from the channel with one and five glutamate molecules attached. Dudel et al. (1993) found that, like the case of Heckmann and Dudel (1997), the rate constant of desensitization from the partly liganded state was much larger than from the fully liganded state.

By contrast, in our kinetic scheme, the rate constant of desensitization from the partly liganded state is weaker than from the fully liganded state.

Another difference between the results of Dudel et al. (1993) and our results is that we measured a much slower time constant of desensitization and resensitization. The extremely fast kinetics reported by Dudel et al. (1993) are not typical of most channels studied.

Comparing the kinetic models for the two crayfish channels and the Drosophila channel reveals structural (the number of glutamate binding sites) and kinetic (rate constant values) differences, but the general form of the three models is similar: all are basically cyclic and include desensitization from a partly liganded channel.

Desensitization from a partly liganded state was also proposed for vertebrate non-N-methyl-D-aspartate (NMDA) glutamate channels (Hauser and Roth 1997; Heckmann et al. 1996; Jonas et al. 1993; Raman and Trussell 1995). Thus it can be considered as a common feature of non-NMDA glutamate channels.

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