Rapid activation, desensitization, and resensitization of synaptic channels of crayfish muscle after glutamate pulses

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ABSTRACT Completely desensitizing excitatory channels were activated in outside-out patches of crayfish muscle membrane by applying glutamate pulses with switching times of ~0.2 ms for concentration changes. Channels were almost completely activated with 10 mM glutamate. Maximum activation was reached within 0.4 ms with ≥1 mM glutamate. Channel open probability decayed with a time constant of desensitization of 2 ms with 10 mM glutamate and more rapidly at lower glutamate concentrations. The rate of beginnings of bursts (average number of beginnings of bursts per time bin) decayed even faster but approximately in proportion to the glutamate concentration. The dose-response curve for the channel open probability and for the rate of bursts had a maximum double-logarithmic slope of 5.1 and 4.2, respectively.

Channels desensitized completely without opening at very low or slowly rising glutamate concentrations. Desensitization thus originates from a closed channel state. Resensitization was tested by pairs of completely desensitizing glutamate pulses. Sensitivity to the second pulse returned rapidly at pulse intervals between 1 and 2 ms and was almost complete with an interval of 3 ms.

Schemes of channel activation by up to five glutamate binding steps, with desensitization by glutamate binding from closed states, are discussed. At high agonist concentrations bursts are predominantly terminated by desensitization. Quantal currents are generated by pulses of >1 mM glutamate, and their decay is determined by the duration of presence of glutamate and possibly by desensitization.

INTRODUCTION

Excitatory transmission at neuromuscular junctions of arthropods is usually mediated by glutamatergic synapses of the quisqualate type (Kawagoe, Onodera and Takeuchi 1984). The postsynaptic channels activated by glutamate have been well characterized in locust (Patlak et al., 1979; Cull-Candy et al., 1981; Cull-Candy and Parker, 1982; Kerry et al., 1987, 1988) and crayfish (Franke et al., 1983, 1986*a*; Franke and Dudel, 1987; Dudel and Franke, 1987; Hatt et al., 1988*a* and *b*). One characteristic of these channels is their very rapid desensitization. Long-term application of glutamate elicits almost no activity in locust muscle, unless desensitization is blocked by concanavalin A, as done in the studies cited above. In crayfish, after initial desensitization, glutamate sustains channel activity in the steady-state. We have shown recently that this steady-state activation is based on channel types which desensitize incompletely. Other channel types show rapid and complete desensitization, like the channels in locusts (Dudel et al., 1990). The desensitizing channels can be studied properly only with pulse application of glutamate, e.g., with the liquid-filament switch that directs glutamate pulses to outside-out patches (Franke, et al., 1987; Dudel et al., 1988*a* and *b*; Dudel, J., Ch. Franke, and H. Hatt, manuscript in preparation). In such recordings high concentrations of glutamate effect maximal opening of channels within <1 ms and desensitization with apparent time constants of 1–10 ms in crayfish, and 25 and 3 ms, respectively, in two channel types in locusts.

Extremely rapid desensitization may be a general characteristic of some quisqualate types of channel. Trussell et al. (1988) and Hatt et al. (1989) saw whole-cell and single-channel currents, respectively, in spinal neurons of embryonic chicken which decayed after application of glutamate with half-times of 3–15 ms. In hippocampal neurons of embryonic or neonatal rats, channel activity elicited by glutamate was inactivated in 3–8 ms (Trussel et al., 1988; Mayer and Vicklicky, 1989; Tang et al., 1989).

It appears that for these channel types the kinetics of desensitization are almost as important as those of activation. Reaction schemes of desensitization of channels at the vertebrate endplate have been discussed since Katz and Thesleff (1957) and have generally assumed that desensitization starts from the open state (see Daoud and Usherwood, 1978; Feltz and Trautmann, 1982; Colquhoun et al., 1989). However, these concepts were based on desensitization experiments using relatively slow switching of agonist concentration. Magleby and Palotta (1981) studied desensitization by eliciting endplate currents with short intervals. They concluded that ACh initiated a desensitization step of the closed channel with time

constants <30 ms. In the present study, a completely desensitizing, glutamatergic channel of crayfish muscle was activated by brief glutamate pulses, and desensitization and resensitization were analyzed. The aim was to test whether desensitization started from an open or a closed channel state and to develop new concepts, if necessary. Pulse application of glutamate may also mimic the situation in synaptic transmission, and the conditions of rise and decay of synaptic current and the possible contribution of desensitization may be analyzed.

METHODS

The experiments were done on muscles isolated from crayfish (*Austropotamobius torrentium*) 3–5 cm in length. The muscles were mainly the abductor (opener) of the dactyl of the first walking leg and the intrinsic stomach muscles of the gastric mill 5b, 6a, and 8b (Maynard and Dando, 1974). The solution superfusing the muscle and the outside of the patch contained 220 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 10 mM Tris-maleate buffer, with the pH adjusted to 7.6 with NaOH. On the intracellular side of the outside-out patches, the pipette was filled with 150 mM K-propionate, 5 mM Na-propionate, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM ethyleneglycol-bis-(2-amino-ethyl tetracetic acid (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 adding KOH. Total K^+ concentration was 190 mM. The temperature was held at 18°C.

The muscles were treated with 0.5 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO; Ia) to remove connective tissue. GΩ-Seal patches could be isolated on these muscles and excised, outside-out patches were prepared as described by Hamill et al. (1981). The procedures and conditions are described in more detail in Franke et al. (1986*a*) and Dudel and Franke (1987).

Patch-clamp currents were recorded with a Neher-Sigworth patch-clamp amplifier (model EPC 7; List Industries Inc., Matteson, IL). The data were stored on video tape (modified Sony PCM-501 ES). They were digitized for processing at 50 KHz and evaluated on a series 300 microcomputer (Hewlett-Packard Co., Palo Alto, CA) using the programs described in Dudel and Franke (1987). For some evaluations "idealized tracks" were constructed from the original recordings. In a first step, in the current record data points which did not deviate from neighboring points by more than a threshold value (e.g., 0.3 pS) were replaced by their average values. After a deviation by more than threshold, a new average was formed. These average values were entered into amplitude histograms (see e.g., Franke et al., 1986*b*, Fig. 6) in which the zero current and channel currents were expressed as distinct peaks. The interpeak minima were selected as limiting values classifying amplitude bins. In a final step, all current values were converted to the average amplitude associated with the respective amplitude bin. Evaluations of data from 41 patches were included in this study.

The liquid filament switch used to apply pulses of glutamate to the excised, outside-out patch is sketched in Fig. 1 (see also Franke et al., 1987). A steel tube contains a polyethylene tube with an opening of ~30 μm diameter. Agonist (usually glutamate)–containing solution is ejected by pressure from this tube and forms a well-defined, thin filament of fluid in a bulk superfusion flowing from right to left. The steel tube is fixed to a piezo crystal (minitranslator P 810.10, Physik Instrumente, Waldbronn, FRG) which, on application of 100 V, moves the tube upward (in parallel) by 20 μm. The shift of the tube was measured with magnetic field plates, and the lower trace of the graph in Fig. 1 shows the time course of this movement. The change from 10 to 90% amplitude (from 2 to 18 μm) took <0.2 ms.

The pipette with the excised patch of membrane at its tip is located ~10 μm above the liquid filament (with 0 V at the piezo) close to the tip of the tube using a micromanipulator. Upon application of 100 V to the piezo, the filament containing agonist is shifted upwards and hits the patch, whereas at the end of the voltage pulse the filament swings away from the patch, and superfusate (control solution) washes the patch again. One way to measure the time course of the displacement of the filament is to use an electrode without a membrane patch, to apply 1:1 diluted control solution through the tube, and to drive a current from the pipette to the bulk solution. Switching the piezo, this current changes as a function of conductivity of the solution at the tip. The upper trace of the graph in Fig. 1 shows the time course of such a current change; it takes 0.2 ms from 10 to 90% of the change. With optimum location of patch and liquid filament, switching times for changes of agonist concentration are therefore in the range of 0.2 ms.

The experiment shown in Fig. 2 demonstrates the quality of the switching for a real patch upon application of 10 mM glutamate (see also Franke et al., 1987). In the left-hand column of records, the piezo was activated for 20 ms at a repetition rate of 1/s. Channels are seen to open directly after the switching artefact and to form bursts with an average duration of 4.1 ms, as shown in the distribution of burst lengths. These channels represent the incompletely desensitizing type (Dudel et al., 1990). The lowest graph is an average of such recordings and shows desensitization with a time constant of ~5 ms. In the right-hand column, the piezo was activated only for 0.4 ms. It is obvious that the bursts of channel openings were shortened due to the brief application of glutamate. However, the average current reached the same maximum amplitude as that measured for 20-ms pulses. There was, therefore, full activation of the channels during the 0.4-ms pulse, and the glutamate was removed from the patch rapidly enough to shorten the bursts of channel openings. It should be noted that the average current for the 0.4-ms glutamate pulses rises faster and decays more rapidly than a synaptic quantal current.

RESULTS

Activation and desensitization

In the present study, outside-out patches of muscle membrane were selected to contain only fully desensitizing channels. We chose this type because for incompletely desensitizing channels it cannot be excluded that the population contains some completely desensitizing ones. Typical recordings of channels activated by pulses of glutamate using the liquid filament switch are shown in Fig. 3. The glutamate pulses lasted 100 ms each and were repeated at a rate of 1/s. Channels opened only for a few milliseconds after switching on the glutamate. During the rest of the pulse and at its end (not illustrated) no channel activity was seen. In the left-hand column of Fig. 3, the high concentration of 10 mM glutamate opened three channels in almost all traces. The channels opened in "bursts," flickering between open and closed states for ~1 ms in each burst. A burst was considered to be terminated if a closed interval ≥0.5 ms occurred (Dudel and Franke, 1987). The burst structure cannot be readily analyzed in

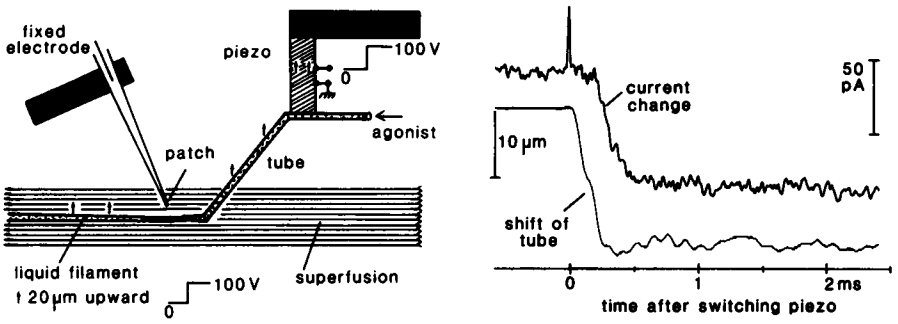


FIGURE 1 Scheme of liquid filament switch for pulse application of agonist to an excised patch, usually in the outside-out mode (pulse superfusion of a small cell attached to the patch-clamp pipette is also possible). Description in text. (*Lower graph*) Time course of parallel shift of the tube on applying 100 V to the piezo crystal, measured with a field-plate attached to the tube. (*Upper graph*) Time course of a change in current between patch-pipette (without patch) and the general superfusate produced by switching the liquid filament, which in this case contains diluted ionic solution.

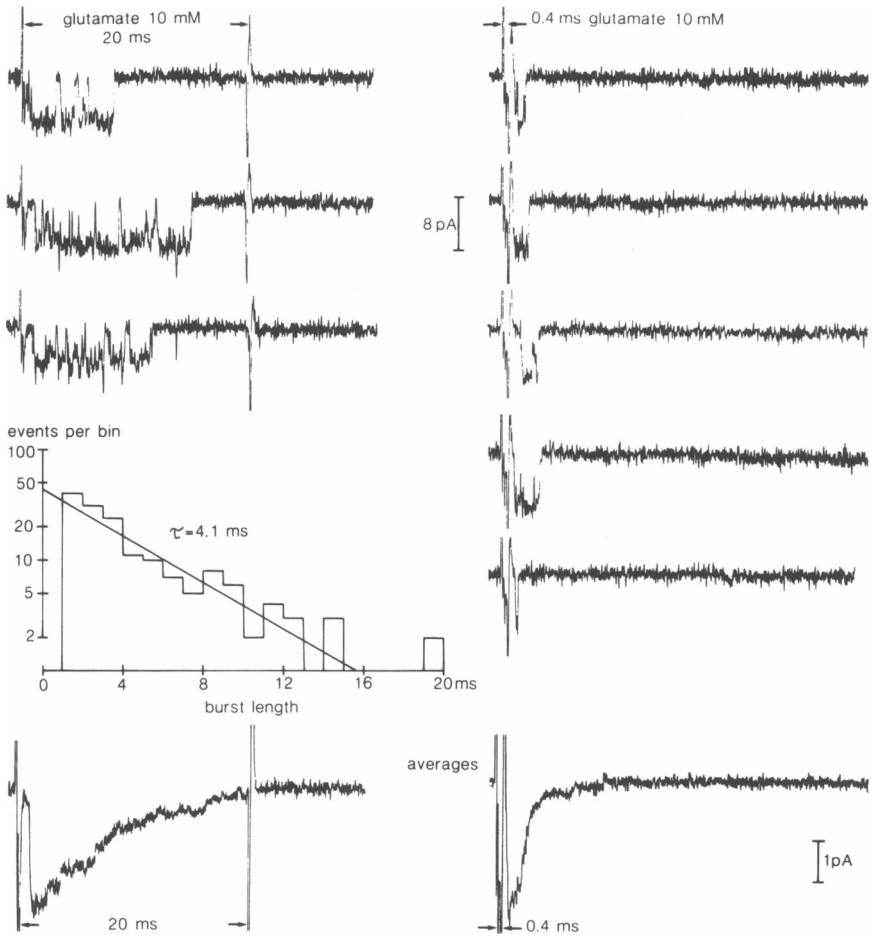


FIGURE 2 Effects of applying 10 mM glutamate to an outside-out patch in pulses of 20 ms (*left*) and of 0.4 ms (*right*) duration. The patch contained an incompletely desensitizing channel (only in this figure). On left, below the three specimen records, a distribution of burst lengths with an average of 4.1 ms. At bottom, an average of 80 recordings like those shown above. The average current exhibits desensitization with a time constant of ~6 ms. On the right, after the 0.4-ms pulse of glutamate the bursts of channel openings are cut off, and the average current decays much more rapidly than during the 20-ms pulse. However, the same maximum average channel current is reached for the short and long glutamate pulses. Low-pass filter at 10 kHz.

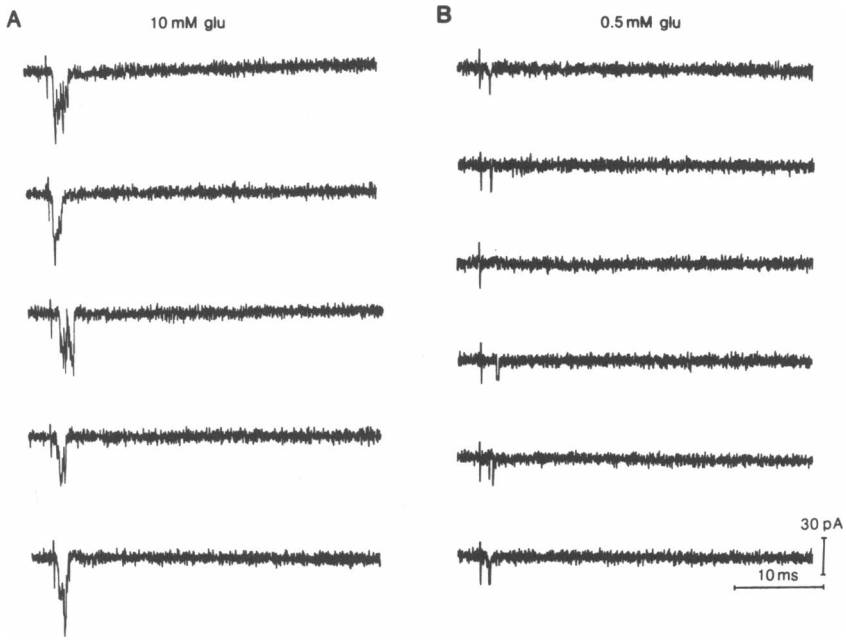


FIGURE 3 Openings of completely desensitizing channels after 50-ms pulses of glutamate to an outside-out patch. (*A*) Glutamate concentration 10 mM; (*B*) glutamate concentration 0.5 mM. With 10 mM glutamate, in almost all trials three channel openings occurred during the first milliseconds, but never four openings. The patch therefore contained three glutamatergic channels. At 0.5 mM glutamate, pulses often failed to elicit openings (third trace, zero responses are underrepresented), and only one channel opening in the other trials. Low-pass filter at 10 kHz. Patch potential, −80 mV.

recordings containing only superimposed bursts. Similar bursts were evaluated in Dudel, J., Ch. Franke, and H. Hatt (manuscript in preparation) in patches containing only one channel. The bursts elicited with 10 mM glutamate had an average duration of 1.5 ms and the single openings lasted on average 0.3 ms, with interspaced closings of on average 0.1 ms.

The fact that almost all pulses elicited opening of three channels with 10 mM glutamate allows a determination of the number, n, of channels present in the patch. Fig. 4 shows a distribution of the number n_x, of channels opening after each pulse. In 100 trials, always at least one channel opened, and opening of four channels was never observed. The measured distribution is fitted exactly if a binomial distribution with $n = 3$ and $p = 0.89$ is assumed. If one assumes $n = 4$ and an appropriately reduced $p = 0.67$, a probability of occurrence of four channels of $p_4 = 0.2$ is predicted, and p_2 and p_3 are of similar magnitude, 0.29 and 0.4, respectively. This distribution is so different from the measured one, that $n = 4$ and higher values of n are clearly excluded. We conclude that the patch of the experiment of Fig. 3 contained three glutamatergic channels.

After pulses of 0.5 mM glutamate (right-hand column in Fig. 3) channels opened much less frequently; in many pulses no channel openings were elicited (underrepre-

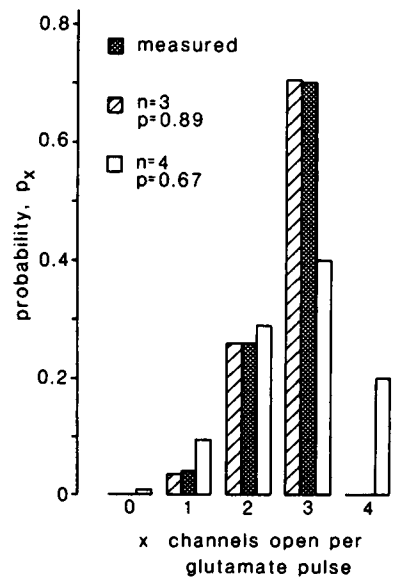


FIGURE 4 Distribution of number, n_x, of channels activated by 10 mM glutamate pulses in the experiment illustrated in Fig. 3 (*stippled bars*). The results of 100 pulse application were evaluated. For comparison, the n_x predicted by a binomial distribution with a total number of channels $n = 3$ and an opening probability $p = 0.89$ (*hatched bars*), and with $n = 4$ and $p = 0.67$ (*open bars*) are plotted.

sented in Fig. 3). The openings were short, and bursts of grouped openings were rare. The shortening of burst length with decreasing glutamate concentration is generally observed in glutamatergic channels (Dudel and Franke, 1987; Dudel et al., 1990). The single-channel current amplitude was ~ −8 pA, corresponding to a channel conductance of ~100 pS (Hatt et al., 1988*a*).

Recordings from the experiment of Fig. 3 were further analyzed by deriving "idealized tracks" (see Methods) and forming their averages. The left-hand column of Fig. 5 shows time courses of channel "open probability" after pulses of three different glutamate concentrations. The ordinate values were derived from the average number of open channels present at a certain instant of time, divided by three, because three glutamatergic channels were present in this patch (Fig. 4). The open probability here therefore gives the absolute probability for a channel to be open. With 10 mM glutamate the open probability was maximally 0.6 (see also Fig. 9).

An open probability of 0.6 is close to saturation, or activation of all channels. In a burst of channel openings, the proportions of average open and shut times are 2:1 to 3:1 (Dudel and Franke 1987; Dudel et al., 1990). Assuming a proportion of open time of 0.7, the theoretical maximum open probability would be 0.7. It follows that in Fig. 3 at 10 mM glutamate 0.6/0.7 ≈ 0.9 of maximally possible channel activation was attained. This value agrees with the probability of opening of 0.89 deduced from the distribution of channel superpositions in Fig. 4. Maximal activation of the channel opening decreased with lower glutamate concentrations, as will be discussed with the dose-response curve presented below.

Switching the glutamate-containing filament produced channel openings with a latency of 0.5–0.6 ms, as can be seen in the evaluations of Fig. 5. Such a latent period was also visible in the "current change" when testing the liquid filament switch without a patch (Fig. 1). This latent period is probably "dead time" of the switch and largely represents the time of flow in the filament between the tip of the tube and the tip of the patch-clamp pipette. After the latent period, the peak of channel acitvation is reached within 0.4 ms for the 10 and the 1 mM glutamate pulses. It should be noted that the rise time is not shorter with 10 mM glutamate than with 1 mM glutamate. Possibly the minimum rise time of 0.4 ms is limited by diffusion between liquid filament and the patch. However, it is also conceivable that the rate of rise is limited by channel activation kinetics close to saturation. With 0.5 mM glutamate the maximum open probability is reached after 0.6–0.7 ms, demonstrating the expected slowing of activation at lower concentrations.

After maximal activation, channel open probability declines to zero within <10 ms in the maintained presence of glutamate. The apparent time constants of this desensi-

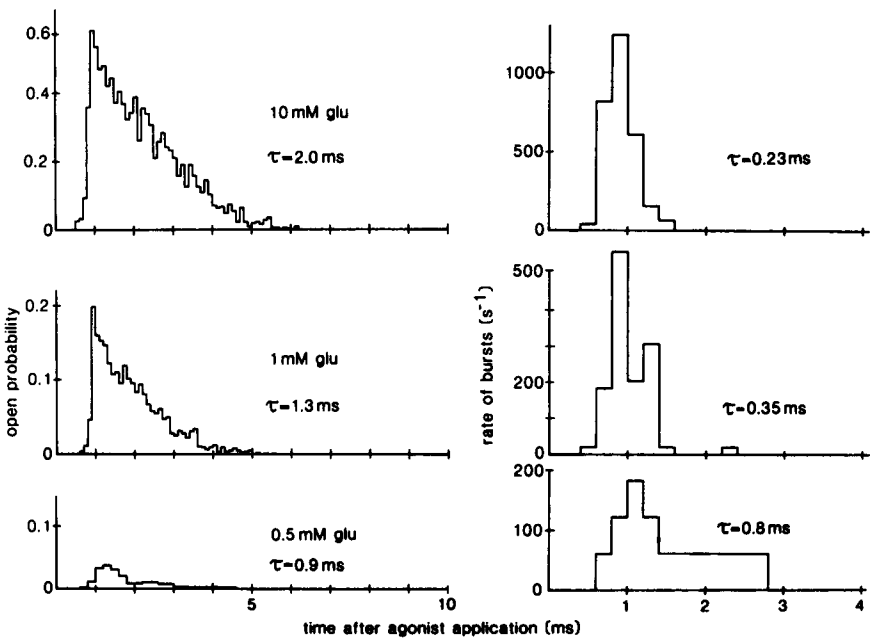


FIGURE 5 Evaluations of open probability (probability of one channel to be open) during 0.1-ms intervals, and rates of beginnings of bursts of channel openings (per channel), for 10, 1, and 0.5 mM glutamate pulses, from the experiment of Fig. 3. The average contained the results of 100 pulses at 10 mM glutamate, of 100 pulses with 1 mM glutamate, and of 60 pulses with 0.5 mM glutamate. The approximate decay time constants are marked at the respective graphs.

tization are 2.0 ms with 10 mM glutamate, 1.3 ms with 1 mM, and ~0.9 ms with 0.5 mM glutamate. These time constants are extremely short, even compared with the 5-ms time constant of desensitization for the mixed channel types (Franke et al., 1987). Furthermore desensitization becomes apparently more rapid on decreasing glutamate concentration, contrary to expectations from simple reaction kinetics.

The anomalous concentration dependence of desensitization rate may be attributable to the fact that the time constant of decay of current through open channels expresses two processes, the decreasing activation of channels, i.e., desensitization, and the distribution of burst durations. With 10 mM glutamate, a population of bursts synchronized to start at zero time will result in an average current decreasing with the time constant of 1.5 ms (Dudel et al., 1990, Fig. 6). Thus the 2-ms decay time constant of the open channels after the 10 mM glutamate pulse in Fig. 5 may be produced by a short pulse of activation of bursts folded with the time constant of 1.5 ms of the distribution of burst durations. With lower concentrations of glutamate, average burst duration decreases (Fig. 3). This decrease in burst duration with decreasing glutamate concentration may mask the true concentration dependence of desensitization. Therefore, the rate of beginnings of bursts was evaluated for the experiment of Figs. 3 and 4. This rate does not contain the burst duration and may serve as a more direct assessment of the rate of desensitization.

The rate of bursts is plotted in the right-hand column of Fig. 5 for the same data represented as channel open probability on the left. Because there are fewer beginnings of bursts than openings of channels, the resolution of the right-hand plots is not as good as that of the left-hand ones. The rising phases of the average burst rates are about the same as those of the open probability, as expected. In contrast, the rates of bursts decay much more rapidly than the channel open probability. With 10 mM glutamate, the approximate time constant of decay of the rate of bursts was 0.23 ms, and this time constant increased to 0.35 ms and 0.8 ms with 1 and 0.5 mM glutamate, respectively. It appears, therefore, that the rate of desensitization increases with increasing glutamate concentration, as expected, if desensitization is caused by binding of glutamate.

It should be noted that with 10 mM glutamate the decay of the rate of bursts expresses not only desensitization but also the effects of saturation of activation. During this decay, between 1 and 2 ms, the open probability drops from 0.6 to 0.35, corresponding to a proportion of 0.9 to 0.5 of channel activation. Therefore the number of channels available for beginning a burst is restricted during this period. With 1 mM glutamate this restriction by saturation is lower by a factor of 3 and therefore much less significant. Formally one could compensate the distortion due to the approach to saturation by dividing the rate of bursts by the proportion of available channels, 0.7 (open probability) (Fig. 5), to obtain a "burst beginning probability." This burst probability would decay even faster (time constant ~0.15 ms) than the burst rate with 10 mM glutamate, but with 1 mM or lower glutamate concentrations its time course would almost be the same as that of the burst rate.

Eleven experiments in which different concentrations of glutamate were applied in pulses had similar results to those presented in Figs. 3 and 4.

Dose-response curve

Fig. 6 *A* shows a plot of maximal open probability, as determined in Fig. 5, against glutamate concentration on a double logarithmic scale. With 10 mM glutamate this dose-response curve approaches the theoretical saturation level of ~90% (see above). Between 0.1 and 0.5 mM glutamate the slope is very steep, attaining a value of 5.1. The response rises in this concentration range by almost four orders of magnitude. With 0.1 mM glutamate, only six channel openings were observed with 1,000 glutamate pulses, and these were distributed over 20 ms. Thus the value with 0.1 mM glutamate is a rough estimate,

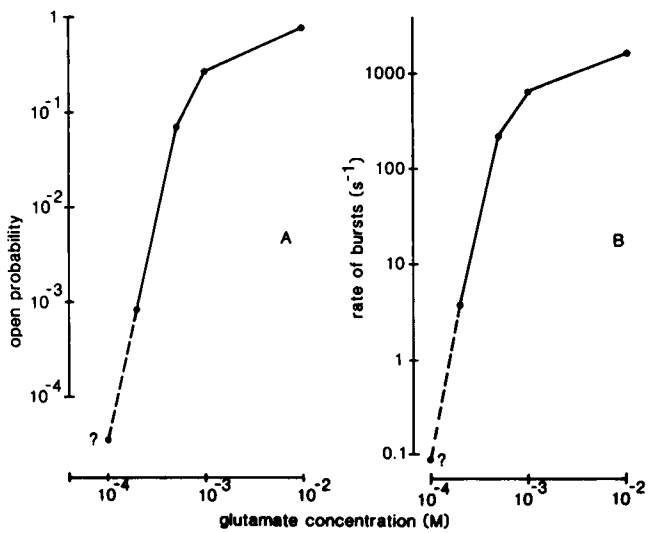


FIGURE 6 Dose-response curves for the activation of completely desensitizing channels by glutamate pulses. Maximum amplitudes of the time courses of open probability (*A*) and of rate of bursts (*B*), as shown in Fig. 5, were plotted in dependence on glutamate concentration for one patch (same experiment as in Figs. 3 and 4). The values with 0.1 mM glutamate are based on relatively little data (see text) and the line to the respective points is dashed to stress their tentative nature. The maximum slopes of the double-logarithmic plots are 5.1 in *A* and 4.2 in *B*.

whereas with 0.2 mM glutamate with almost 100 times higher activity the possible error of the peak value is much lower. The double logarithmic slope of 5 is seen already between 0.2 and 0.5 mM glutamate.

The maximum double-logarithmic slope of the dose-response curve is a lower limit for the power law which describes the concentration dependence of the response. This power, n_H, is often equated to the number of binding steps for glutamate at the receptor necessary to obtain a response (see e.g., Werman, 1969; Colquhoun, 1975). Although this equation may not apply if desensitization reactions have rates of the same order of magnitude as the activation steps, n_H is an important value for the construction of schemes of receptor kinetics. Because the channel open probability reflects not only activation of the channel but also burst duration, with regard to reaction kinetics, the rate of bursts (Fig. 5) is a more direct measure of activation. Therefore, a dose-response curve was drawn for the maximal rates of bursts in Fig. 6 *B*. This graph is somewhat less steep than that in Fig. 6 *A*. The maximal double-logarithmic slope is 4.2.

Desensitization from open or closed channel state

Expanding the scheme proposed by del Castillo and Katz (1957) and Katz and Thesleff (1957), and including the work of Magleby and Stevens (1972), Colquhoun and Sakmann (1985), and others on vertebrate endplate channels, the most probable reaction scheme for the binding of the agonist A to the receptor R has the form:

$$\begin{array}{ccccccc} A & A & & & & & \\ + & + & & & & & \\ R & \rightleftharpoons & AR & \cdots\cdots \rightleftharpoons & A_nR & \rightleftharpoons A_nS & \rightleftharpoons A_nD \\ & & & & \downarrow\uparrow & & \downarrow\uparrow \\ & & & & \cdots\cdots\cdots\cdots & A_{n-1}D + A & \end{array}$$

Scheme 1

In this sheme n is the number of agonists which have to bind to allow a conformation change from the closed state A_nR to the open state A_nS. A_nD is a desensitized state which is reached mainly by a conformation change from the open state, A_nS. Franke et al. (1987) measured the time constant of desensitization as decay of the average current after a glutamate step. Because this time constant was independent of the glutamate concentration (in a mixed-channel population), desensitization should not directly involve binding of glutamate. Thus it probably proceeds from the open state. In the present study of fully desensitizing channels, Fig. 5 (open probability) showed that desensitization even increased in rate on lowering the glutamate concentration. However, when in Fig. 5 desensitization was expressed as decay of the rate of beginnings of bursts, desensitization became more rapid with increasing glutamate concentration. Consequently, desensitization should involve binding of glutamate and could start from any of the R and S states in Scheme 1. This was tested experimentally.

If desensitization started not from an open state, S, but from a closed state, R, it might be possible to drive all channels into the desensitized state without evoking any channel opening by applying low concentrations of glutamate. In the experiment illustrated in Fig. 7, each test pulse of 10 mM glutamate elicited openings of two or three channels followed by rapid, complete desensitization (control records, left column). When a subthreshold concentration of glutamate was added to the continuous superfusion (right column), no openings of channels were seen. Extrapolating the dose-response curve in Fig. 6, ~20 openings/h are expected. 10-mM glutamate test pulses applied on the background of 50 μM glutamate evoked responses very rarely, at ~1/100 of the rate of the controls. Fig. 8 summarizes the results of two such experiments. Whereas 10 μM glutamate in the superfusion of the bath decreased the reaction towards the 10 mM glutamate test pulse only a little, 20 and 50 μM glutamate almost blocked the responses. These results are difficult to reconcile with desensitization from an open state. They are not fully conclusive, however, because the desensitized state could have a long lifetime, and the three channels present in the patch could all be desensitized, even if they had very low opening rates.

The more complicated experimental protocol of Fig. 9 contains a control of the lifetime of the desensitized state. In this experiment the desensitization was effected by a glutamate concentration which slowly rose from 0 to 10 mM. This was achieved by applying a voltage ramp to the

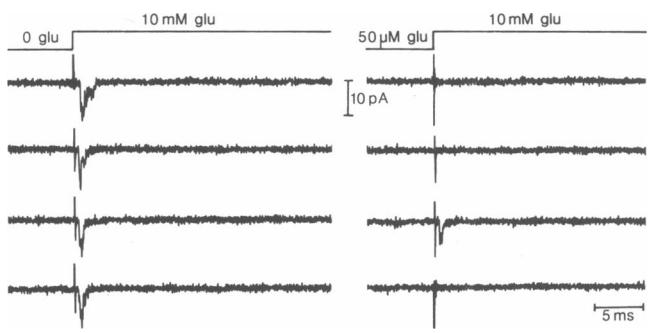


FIGURE 7 Predesensitization experiment. In the controls (*left*) a 10 mM glutamate test pulse elicited two or three channel openings in the first milliseconds of each pulse. When the background, bulk superfusion contained 50 μM glutamate (*right*), the same test pulse caused rare, single openings. No channel openings were seen during superfusion of 50 μM glutamate without test pulses. Filter at 5 kHz, patch potential −80 mV.

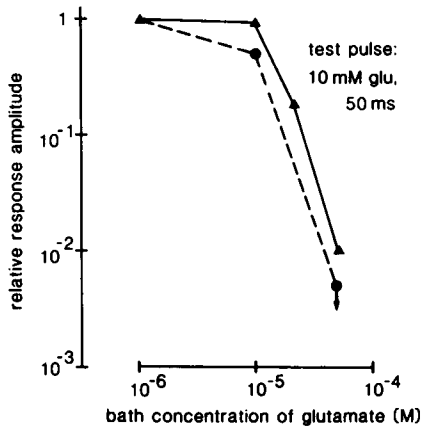


FIGURE 8 Evaluation of two experiments like that in Fig. 7. (*Ordinate*) Amplitude of the response to a test pulse of 10 mM glutamate (maximum number of open channels) in relation to the response in controls. (*Abscissa*) Predesensitizing concentration of glutamate in the bulk superfusate. The value at 0.5 mM glutamate with an arrow is an upper limit.

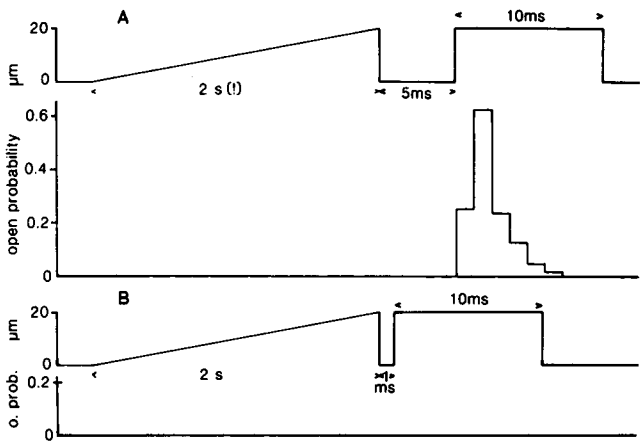


FIGURE 9 Desensitization with a slowly rising glutamate concentration. In *A* and *B* the upper plots show the displacement of the tube ejecting the liquid filament. At zero position, the filament passes below the patch, the patch being washed by 0 glutamate solution in the bulk superfusion. Then the liquid filament which contains 10 mM glutamate is moved upward with a ramp voltage which causes the full 20-µm excursion of the piezo within 2 s. During this ramp the concentration gradient in the liquid filament from 0 to 10 mM glutamate passes the patch slowly, but does not elicit any channel activation (lower traces in *A* and *B*). At the end of the voltage ramp, the voltage at the piezo is switched to zero and the patch is shifted back to zero glutamate solution. From this point on, the time scale in the graphs is expanded by a factor of 100. In *A* a test pulse of 10 mM glutamate for 10 ms follows within 5 ms after the ramp. This test pulse activates the three channels present in the patch maximally (*lower trace*). In *B* the same test pulse is given with a 1-ms interval after the ramp. No openings are observed, indicating that during the 1-ms interval the complete desensitization during the ramp had not been removed. The averages of open channels contain the result of 70 glutamate pulses in *A*, and of 60 glutamate pulses in *B*.

piezo which slowly shifted the tube to its end position (20 µm) over a period of 2 s. During this shift the border zone of the liquid filament with its steep gradient of glutamate concentration, from 0 to 10 mM, slowly passed the patch, exposing it to slowly rising glutamate concentrations. This procedure was repeated 30 times, but no channel openings were seen during these ramps. After the ramp was completed, the filament was shifted back rapidly to the control position. The patch was then superfused again by control (0 glutamate) solution. After a fixed short interval, a 10 mM glutamate test pulse was added. If this interval was 1 ms, no channel openings were seen, proving that the channels were desensitized during the ramp and had not yet recovered from desensitization (Fig. 9 *B*). If, however, the interval between ramp and test pulse was 5 ms, the test pulse opened an average of two channels (Fig. 9 *A*), which was as many as in the control without a previous ramp (not illustrated). We do not know the exact time course of the change in glutamate concentration at the patch during the ramp, but this is not essential for the present purpose; it suffices that the channels are shown to be desensitized finally and that no openings are observed during the ramp. This experiment (Fig. 9) demonstrates unambiguously that desensitization can be produced without channel opening.

Resensitization after desensitization

The experiment illustrated in Fig. 9 gave a first example of the rapid resensitization process of the channels studied here; 5 ms washing of the patch with control solution was enough to reverse complete desensitization. The time

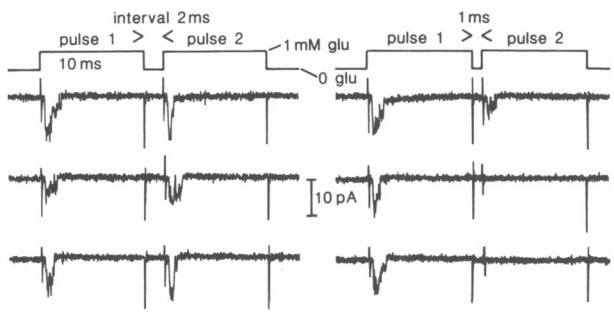


FIGURE 10 Time course of resensitization. Pairs of test pulses containing 1 mM glutamate, each lasting 10 ms, were given with intervals of 2 ms (*left*) and of 1 ms (*right*) to one patch. The pulses elicited openings of maximally three channels and after a few milliseconds complete desensitization. In each pair, pulse 1 is the control, and the response in pulse 2 indicates the extent of removal of desensitization, i.e., resensitization, during the pulse interval. With 2-ms pulse interval the response during pulse 2 had almost control amplitude. With 1-ms pulse interval, the responses to pulse 2 were strongly depressed, no channels opening during most of the trials. Patch potential −80 mV, filter at 5 kHz.

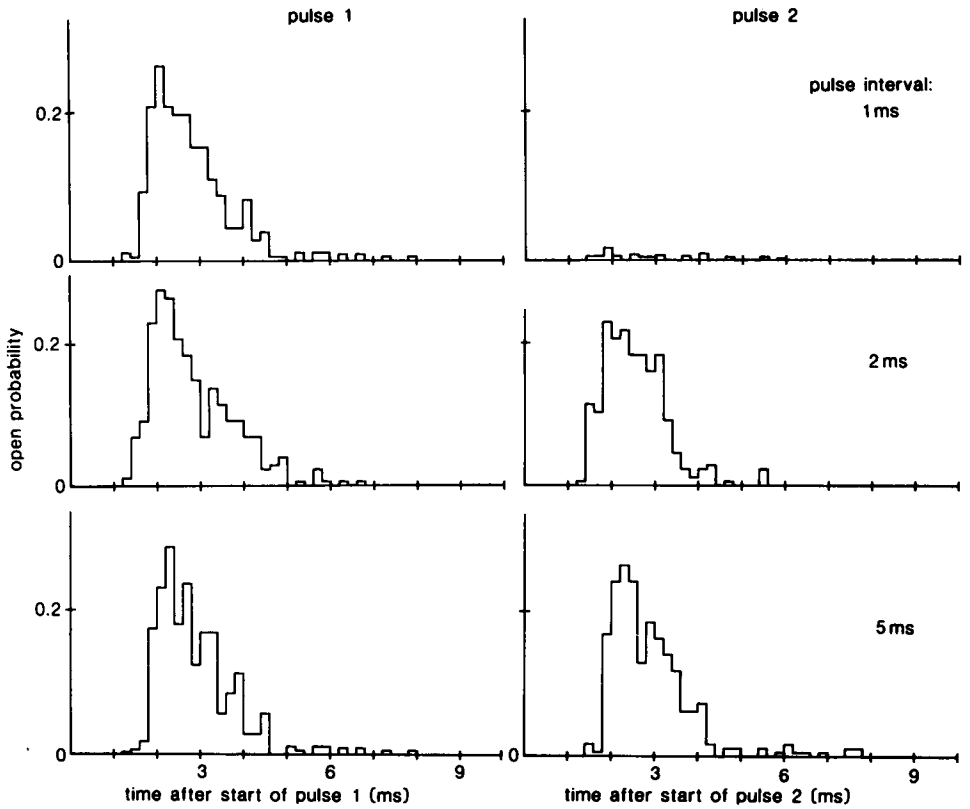


FIGURE 11 Evaluation of the experiment in Fig. 10. Averages of the time course of channel openings after the 1 mM glutamate pulses. The effects of pulse 1 are the controls. Pulse 2 elicits almost no openings with 1-ms interval, almost control responses with 2-ms interval, and approximate control responses with 5-ms interval.

course of resensitization was studied systematically by applying pairs of 10-ms pulses of 1 mM glutamate with varying interpulse intervals. Similar measurements were done by Clark et al. (1979) in locusts and yielded time constants of resensitization of 0.2–10 s for different channels. The example in Fig. 10 shows that with 2-ms pulse intervals, pulse 2 could elicit almost as large a response as pulse 1. In contrast, with 1-ms intervals the responses to pulse 2 were strongly depressed.

Fig. 11 summarizes the channel open probability for intervals of 1, 2, and 5 ms. With 1-ms intervals, channel opening is strongly suppressed; desensitization is still almost complete. Between 1- and 2-ms intervals, the gradient of recovery is very steep, and at 5 ms, interval recovery is complete. Fig. 12 shows a plot of the relative amplitudes of the responses against the pulse interval for three patches. 1 ms after the end of the test pulse which had caused complete desensitization for ~5 ms, the probability of activating resensitized channels was on average only 0.18. With a 2-ms interval, this probability rose to 0.8, and it was close to 1 from 3 ms on. It is very difficult to obtain a higher number of values for the measurement of a more detailed time course of resensiti-

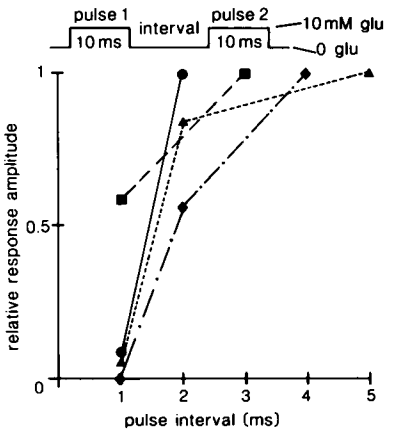


FIGURE 12 Results from four experiments like those in Figs. 9 and 10. Pulse regime is shown at top. (*Ordinates*) Maximal amplitude of open probability like shown in Fig. 11, the response to pulse 2 divided by the response to pulse 1. (*Abscissa*) Interval between pulses 1 and 2.

zation, because the large number of double pulses at high glutamate concentrations stresses the stability of the patch. Thus many experiments are unsuccessful because not enough data can be collected to establish at least two averages in the range of interest. The time course of resensitization is probably S-shaped, with more than half of the resensitization occurring between 1 and 2 ms after the desensitizing glutamate concentration was switched off. Resensitization thus seems to be about as rapid as desensitization.

DISCUSSION

Dose-response curves

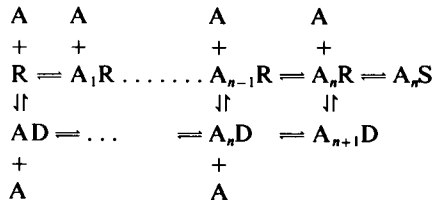
The dose-response curve of the completely desensitizing channels shows strikingly high slopes at glutamate concentrations between 0.1 and 1 mM, amounting to n_H = 5.1 in the double-logarithmic plot of Fig. 6 *A*. The corresponding value for incompletely desensitizing channels was lower, about n_H = 2.5 (Dudel et al., 1990). In crayfish muscle, double-logarithmic slopes for the dependence of macroscopic current on glutamate concentration are 4–6 (Dudel, 1975, 1977; Dekin, 1983). In the vertebrate endplate the slopes were n_H = 2–3 for macroscopic currents (see Dreyer et al., 1978) and channel openings (Colquhoun and Ogden, 1988), respectively. Slopes $n_H > 1$ seem to be typical for the activation of synaptic channels and provide for a threshold of agonist concentration above which the effect rises very steeply.

The maximum slope of the double-logarithmic dose-response curve, n_H, is often used to estimate a lower limit for the number of binding steps, n, necessary to open a channel (Colquhoun, 1975; Werman, 1969; Ogden and Colquhoun, 1985). In the present study, n_H = 4.2 derived from the dose dependence of the rate of bursts may be relevant and indicate an n = 5 for the reaction Scheme 1. However, $n \geq n_H$ applies strictly only for reaction schemes which do not contain desensitization. Depending on the specific mechanism involved, desensitization may increase or decrease the slope of the dose-response curve, and the estimate n = 5 of binding steps of the agonist to the glutamate receptor consequently is tentative in character. Jackson (1989) has shown that at least two agonist binding steps are necessary to provide energy for sufficient agonist sensitivity and speed of channel opening, and this argument sets a lower limit of n = 2.

Desensitization

The rates of desensitization reported here, with time constants of the decay of channel openings of ~1 ms (Fig. 5), are extraordinarily high. Similar high rates have been measured for quisqualate-type channels after pulse application of glutamate also in locusts (Dudel et al., 1988*b*) and in central neurons of vertebrates (Trussell et al., 1988; Hatt et al., 1989; Mayer and Viklicky, 1989; Tang et al., 1989). As mentioned in the introduction, the high rates of desensitization may be a specific feature of quisqualate-type channels. Measurements of macroscopic currents, with pulses of glutamate superfusion of crayfish muscle, yielded time constants of desensitization of ~900 ms at 0.5 mM glutamate (Dudel, 1977; see also Clark et al., 1979, for locust) which are two to three orders of magnitude longer than the ones recorded now. These differences probably are due to the unavoidably slow solution changes in bulk superfusions. Feltz and Trautmann (1982) found a 'short' time constant of desensitization of 1 s for the vertebrate endplate, also using a bulk superfusion. This value is also probably much too long (see Dreyer et al., 1978). Magleby and Palotta (1981) concluded a two-step desensitization mechanism with binding of ACh, the first step proceeding with a time constant of <30 ms. Brett et al. (1986) reported a 50-ms desensitization time constant for cholinergic channels at a cultured nerve cell. Employing a rapid fluid exchange system at outside-out patches, Maconochie and Knight (1989) found time constants of desensitization between 15 and 50 ms in nicotinic channels from membranes of a muscle cell line and of chromaffine cells. As discussed below, at vertebrate endplates time constants of desensitization in the range of 10 ms seem probable.

We have shown in the experiments of Figs. 5–8 that desensitization is due to binding of glutamate to a closed state of the channel. Scheme 1 of desensitization, from which this investigation started, thus has to be altered to a scheme of the following type:



Scheme 2

in which D is a desensitized state. In Scheme 2 desensitization is thought to occur mainly from A_nR, but it could occur from any other of the R states. Scheme 2 is fairly general, but it excludes desensitization steps without binding of agonist. An equilibrium between R and D states due to spontaneous conformational changes has been discussed by Katz and Thesleff (1957). A significant proportion of channels in a D state which are not immediately available for activation seems to be excluded by the

present results, because a 10-mM glutamate pulse can drive 90% of the three channels present in the patch into the A_nR and A_nS states within 0.4 ms (Fig. 5). If a variable number of channels would be unavailable for activation in the D state during these 0.4 ms, a fixed ceiling of three channels activated would not be observed.

Apart from inactivation by agonist binding to a closed state, a scheme of desensitization should account for the following characteristics of the completely desensitizing channel: (*a*) After a rapid rise in glutamate concentration to 1 or 10 mM, the first activations should pass the chain of reactions to reach the A_nS open state for 90% of the channels within <0.4 ms. (*b*) Activations are blocked with a time constant of ~0.3 ms after maximal channel opening. (*c*)After removal of 1 mM glutamate, within 3 ms all desensitized channels are back to activatable R states (Figs. 10–12). (*d*) In addition to the specifications given by the time courses of activation, desensitization, and resensitization, a reaction scheme should account for the high slopes of the dose-response curves (Fig. 6). With forward and backward rates of binding steps of agonist leading to channel opening and to desensitization, all apparently of similar magnitude, it appears to be difficult to achieve a high slope of the dose-response curve.

Scheme 2 is probably insufficient to describe this combination of features of desensitization. Quantitative modeling is necessary to see the effects of different combinations of rate constants in Scheme 2 on channel behavior, and to explore extensions of the scheme. An improved model may contain a two- or three-step cooperative reaction leading to desensitization, for this could account for a time lag of desensitization and a delayed steep switching from activation to desensitization. The time course of resensitization (Fig. 12) also seems to indicate an S-shaped temporal switching behavior. Desensitization at glutamatergic channels shares many characteristics with inactivation of potential dependent Na^+ channels. Possibly there are also analogies in the molecular mechanisms.

The present study used only the completely desensitizing glutamatergic channel. However, activation times and desensitization time constants of the incompletely desensitizing channels are in the same range as those of the completely desensitizing channels (Dudel et al., 1990). Predesensitization experiments (Figs. 7–9) are difficult to interpret in incompletely desensitizing channels. Double-pulse experiments testing resensitization (Figs. 10–12) in incompletely desensitizing channels show full recovery of activation within a few milliseconds after the end of a desensitizing glutamate pulse. Probably the two types of channels differ only in the quantitative relations of time constants.

Quantal synaptic currents and desensitization

How does the time course of quantal synaptic currents, of EPSCs, relate to the results on the kinetics of activation and desensitization of the channels? The rise time of EPSCs at 18°C is as short as 0.5–0.6 ms (10–90% amplitude; see Dudel et al., 1988, Fig. 3). To reach such a rise time with a glutamate pulse, the concentration has to be >1 mM (Fig. 5). This way of estimating the lower limit of the concentration of transmitter acting at the receptors seems to us the only valid one so far. For a vertebrate endplate, Land et al. (1981) report an even shorter rise time of EPSCs of 0.2 ms (see also Parnas et al., 1989). Together with the forward rate of activation by acetylcholine of $10^8\ M^{-1}s^{-1}$ given by Colquhoun and Sakmann (1985), this high rate of rise of the EPSC would require acetylcholine concentrations >100 μM, in agreement with the estimate of Land et al., (1981). In the simulation of Parnas et al. (1989) the ACh concentration went up to 2 mM.

The situation during the decay phase of a synaptic current is substantially different for nicotinic and glutamatergic channels. This difference is a consequence of the burst kinetics of the two channel types at low agonist concentrations. If one considers only the burst-generating last steps of the channel opening reaction and disregards desensitization, Scheme 2 gives

$$\ldots A + A_{n-1}R \underset{k_{-n}}{\overset{k_{+n}}{\rightleftharpoons}} A_nR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_nS$$

Scheme 3

Bursts of channel openings occur primarily by oscillations between the states $A_nR \rightleftharpoons A_nS$, being terminated by dissociations $A_nR \rightarrow A_{n-1}R + A$. The "primary burst" is extended to a "secondary burst" if binding reactions $A + A_{n-1}R \rightarrow A_nR$ compensate some of the dissociations. Operationally, the burst is defined as a series of openings which is terminated by a closed period significantly longer than the intraburst closings. The burst-limiting interval was set at 0.5 ms in nicotinic and glutamatergic channels. Binding reactions can contribute to the burst if a significant number will occur during this period of 0.5 ms. The binding rate was $k_n = 10^8\ M^{-1}\ s^{-1}$ in Colquhoun and Sakmann (1985), which is close to the diffusion limit and may be assumed for the glutamatergic channels, too. This rate results in one binding in 0.5 ms at [A] = 20 μM. With [A] $\ll$ 20 μM, therefore, primary bursts are generated. Once the channel opens the average length of a burst is (Colquhoun and Hawkes, 1981, Eq. 2.23):

$$\tau_b([A] \rightarrow 0) = (1 + \beta k_{-n}^{-1})\alpha^{-1} + \beta k_{-n}^{-1}(\beta + k_{-n})^{-1} \quad (1)$$

When the values of $\alpha = 714\ s^{-1}$, $\beta = 30{,}600\ s^{-1}$ and $k_{-n} = 16{,}300$ are taken from Colquhoun and Sakmann (1985) for channel opening by ACh, τ_b ([A] $\rightarrow$ 0) = 4.1 ms results, which agrees with experimental findings. In the channels of crayfish, low glu concentrations elicit mostly single openings (Fig. 3; Dudel and Franke, 1987, Figs. 1 and 3). With the values $\alpha = 5{,}000\ s^{-1}$ and $\beta = 16{,}000\ s^{-1}$ from the latter publication, a high value of $k_{-n} = 100{,}000\ s^{-1}$ has to be chosen to reach a τ_b ([A] $\rightarrow$ 0) = 0.23 ms. The high value of k_{-n} is plausible because n binding sites are available for dissociation. The high dissociation rate may also be necessary for rapid recovery from desensitization.

To generate an EPSC in the nicotinic synapse, therefore, channels have to be opened by a short (100-μs) pulse of high ACh concentration, and the opened channels will perform low-concentration primary bursts of on average 4 ms duration, even if ACh concentration falls immediately to zero. In the glutamatergic channels, a 100-μs pulse of 10 mM glu may open channels with high probability, but there would be only one opening (Fig. 2), generating a current change much shorter than an EPSC. Glutamatergic EPSCs, therefore, cannot be generated by primary bursts. A high glu concentration has to be maintained at the channels for almost as long as the EPSC lasts, i.e., for >1 ms. This maintained high glu concentration will generate secondary bursts, the length of which increases in proportion to glu concentration by providing an increasing number of binding reactions $A + A_{n-1}R \rightarrow A_n$ during the limiting closed interval of 0.5 ms. Such secondary bursts were also named "clusters" (Sakmann et al., 1980; Colquhoun and Hawkes, 1982; Colquhoun and Ogden, 1988). The length of the secondary bursts will be limited mainly by desensitization, e.g., by the reaction $A_nR \rightarrow A_{n-1}D$ in Scheme 2. At [ACh] $\geq$ 160 μM or [glu] $\geq$ 1 mM, respectively, the rate of generation of A_nR is as high as its dissociation, but due to desensitization bursts are evident in the continued presence of agonist.

In crayfish muscle the time constant of decay of quantal EPSCs varies between 0.5 and 3 ms with an average of 1.4 ms (Dudel and Franke, 1987; Dudel et al., 1990). These time constants are about equal to or longer than the time constant of desensitization of the completely desensitizing channel with 1 mM glutamate. The time constant of desensitization of the incompletely desensitizing channel is ~5 ms with 1 mM glutamate, and this channel may also contribute to the EPSC, being subject to some desensitization. Different mixtures of these channel types in the activated synaptic area may be the basis for the astonishingly large variation in the decay times of individual quantal currents at one synaptic site. Another determinant of the decay of quantal currents may be the duration of the presence of a high glutamate concentration at the receptors. Possibly all these factors—mix of channel types activated, desensitization, and duration of presence of glutamate—contribute to determine the decay of individual EPSCs.

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