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THE EFFECT OF CALCIUM IONS ON THE GLUTAMATE RESPONSE AND ITS DESENSITIZATION IN CRAYFISH MUSCLE FIBRES

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SUMMARY

1. The responses of crayfish muscle fibres to bath application or long ionophoresis of L-glutamate were studied in normal and low Ca^{2+} solutions.

2. The smaller responses recorded in low Ca^{2+} solutions have characteristics suggesting a faster desensitization.

3. Desensitization and recovery have complex kinetics. Desensitization is faster and recovery slower when external Ca^{2+} concentration is reduced. Both components of the recovery phase, which can be fitted by the sum of two exponentials, are affected by the external Ca^{2+} concentration.

4. Recovery can be accelerated by external Ca^{2+} ionophoresis onto desensitized glutamate receptors.

5. Responses to brief glutamate pulses of low intensity are not affected by Ca^{2+} reduction. For higher intensities, signs of desensitization are detectable early in the rising phase of the response.

6. Concanavalin A (Con A) blocks both desensitization and Ca^{2+} dependence with similar time courses.

7. Whether or not the preparation has been treated with Con A, the slowly rising responses recorded in isotonic Ca^{2+} do not show signs of desensitization.

8. Con A causes a partial blockade of the glutamate response.

9. The Ca^{2+} dependence of the glutamate response can be explained by the Ca^{2+} dependence of the desensitization process, the cation acting at ectocellular sites of the muscle membrane.

INTRODUCTION

The observation that L-glutamate depolarizes crustacean muscle fibres (Takeuchi & Takeuchi, 1964) initiated a series of studies assessing and supporting the hypothesis that this amino acid is the excitatory neurotransmitter at crustacean neuromuscular junctions (Kawagoe, Onodera & Takeuchi, 1981, 1982). Many of the pharmacological properties of the glutamate receptors have been described (reviewed by Nistri & Constanti, 1979) and the ionic characteristics of the current flowing through the glutamate-activated channels have been established.

It is now clear that the inward current generating the glutamate response is carried mainly by Na^+ ions, although both Ca^{2+} and Mg^{2+} ions have been shown to be

permeant (Dekin, 1983). Since Na^+ is by far the major cation in crustacean physiological solutions, reducing the normal concentration of Ca^{2+} would not be expected to have a marked effect on the amplitude of the glutamate response. However, several investigations revealed that decreasing external Ca^{2+} considerably reduces the glutamate response, a phenomenon referred to below as the Ca^{2+} dependence of the response (Onodera & Takeuchi, 1976; Ashley & Campbell, 1978; Thieffry & Bruner, 1978; Nickell & Boyarsky, 1980). In contrast, the response to single quanta of the natural transmitter are less affected (Thieffry & Bruner, 1978; Dudel, 1981). This dependence on Ca^{2+} is specific, since compensating for the lowered Ca^{2+} concentration with other divalent cations does not impede the effect of a reduction in external Ca^{2+} (Onodera & Takeuchi, 1976; Ashley & Campbell, 1978; Nickell & Boyarsky, 1980). In fact, adding other divalent cations or increasing the Ca^{2+} concentration causes a depression in the glutamate response, presumably by an action of these ions at the channel level (Barker, 1975).

The recent finding (Thieffry, 1982) that the lectin concanavalin A (Con A), which blocks the desensitization of glutamate receptors (Mathers & Usherwood, 1976; Shinozaki & Ishida, 1978), also eliminates the Ca^{2+} dependence of the response strongly suggests that Ca^{2+} could be involved in the desensitization process. The experiments reported below were thus carried out in order to evaluate if Ca^{2+} affected the desensitization process in such a way that this could explain the Ca^{2+} dependence of this glutamate response.

METHODS

Preparation, solutions and recording

The experiments were carried out on the posterior phasic abdominal flexor of the crayfish *Procambarus clarkii* (Selverston & Remler, 1972). The responses which can be elicited by glutamate ionophoresis onto discrete spots of a few square micrometres are of small amplitude and desensitize very rapidly, presumably because of the small number of receptors clustered at each spot. Because of its characteristic desensitization by small amounts of agonist, this preparation was chosen for the present study. Due to its organisation in subunits in which fibres are electrically coupled by cytoplasmic bridges (Brown & Newby, 1980), voltage clamping of the fibres is difficult. Responses can however be evaluated by the change in membrane potential since no rectification occurs over a wide range of potential (Lehouelleur, 1978) and since at 15 °C, the temperature at which the experiments were carried out, the changes in input resistance during glutamate application are negligible (see Fig. 2*A*).

The preparations, mounted in 1 or 2 ml chambers, were continuously superfused. The standard physiological solution contained 195 mM-NaCl, 5·4 mM-KCl, 13 mM-$CaCl_2$, 2·6 mM-$MgCl_2$, 10 mM-Tris maleate buffer and was adjusted to pH 7·2 with NaOH. Solutions containing lower Ca^{2+} concentrations were obtained by mixing this solution with variable amounts of another solution in which $CaCl_2$ was omitted and replaced by 19 mM-NaCl. The 'isotonic Ca^{2+} solution' contained 5·4 mM-KCl, 152 mM-$CaCl_2$, 2·6 mM-$MgCl_2$, and buffer as above. For bath application, glutamate was added to the perfusion solution. For Con A treatment, the lectin (IBF or Sigma type IV) was dissolved in 50 ml standard saline which was perfused in a closed loop after elimination of the first 10 ml.

Membrane potentials were recorded through 10–15 MΩ electrodes filled with 3 M-KCl and continuously monitored on a Gould 2400 pen chart recorder and oscilloscope. In some cases, the responses were recorded on disks and processed off-line on a PDP 11-03 microcomputer. Recording and ionophoresis electrodes were positioned under the microscope at a ×400 magnification. Glutamate (Merck) and Ca^{2+} were applied ionophoretically using constant current generators of the type described by Dreyer & Peper (1974). Ca^{2+} pipettes of low resistance were filled with a 1 M-$CaCl_2$ solution.

Control of the glutamate ionophoresis

In order to avoid leakage of glutamate from the ionophoretic pipette, only high resistance (150–400 MΩ) electrodes were used, and they were filled with a relatively low concentration of glutamate (0·1 M). Under these conditions, it was possible to obtain long-term stability in response amplitudes. When no braking current was applied to the glutamate pipette, desensitization developed rapidly, even with 200 MΩ electrodes. As can be seen in Fig. 1 (which shows the

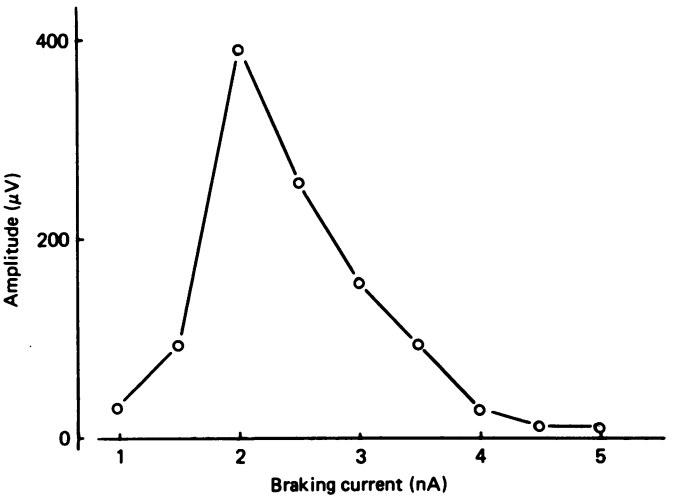

Fig. 1. Amplitude of the responses to ionophoretically applied glutamate at a weakly sensitive spot as a function of the braking current applied to the glutamate pipette. Each point represents the averaged amplitude of twenty responses elicited every 5 s by a 5 ms 120 nA glutamate pulse. The braking current was applied for at least 1 min before each series. The ionophoresis pipette, filled with 0·1 M-L-glutamate at pH 8 had a resistance of 150 MΩ.

relationship between the response amplitude and the braking current applied to a 150 MΩ pipette), adjustment of the braking current was very critical. For braking currents lower than 2 nA, desensitization develops quickly, with a 90 % reduction in response amplitude following a lowering of the braking current by 1 nA. The response modification associated with a 1 nA decrease in braking current is less marked if the braking current is greater than 2 nA. The maximum amplitude is probably that of a response slightly potentiated by a tiny leakage (Takeuchi & Takeuchi, 1964). For the experiments reported below, the braking current was thus adjusted at each spot 0·5 nA above the value which gave the maximum response and remained unchanged throughout the recording. Its value ranged from about 2·5 nA for the lowest resistance accepted (150 MΩ) to 1 nA for the highest resistances compatible with current passing capabilities (of the order of 400 MΩ). Resistances were measured by passing a negative pulse of 10 nA.

RESULTS

Responses to glutamate in standard and low Ca^{2+} solutions

The fibres of fast abdominal flexor muscles respond to bath application of L-glutamate by a depolarizing response. For glutamate concentrations of about 100 μM, the response in standard solution is generally not sustained and declines during agonist perfusion. The amplitude and form of the response depend strongly on the external Ca^{2+} concentration. Responses recorded successively from the same

fibre in the presence of 1·3 mM-, 4·2 mM- and 13 mM-Ca^{2+} are shown in Fig. 2*A*. For the lowest concentration, the response is almost abolished. Ca^{2+} reduction below the standard concentration of 13 mM induces a reduction of both peak and steady-state amplitudes and shortens the time-to-peak. The effect is reversible by washing with the standard solution. It is still observed when the preparation is cooled to 3 °C or when Mg^{2+} is omitted in the bathing solution. When glutamate is applied by 0·1–1 s ionophoretic pulses to sensitive spots of the muscle fibres, the response in low Ca^{2+} solution is similarly characterized by lower peak and steady-state amplitudes and by shorter time-to-peak (Fig. 2*B*). By contrast, the initial slope is clearly unchanged.

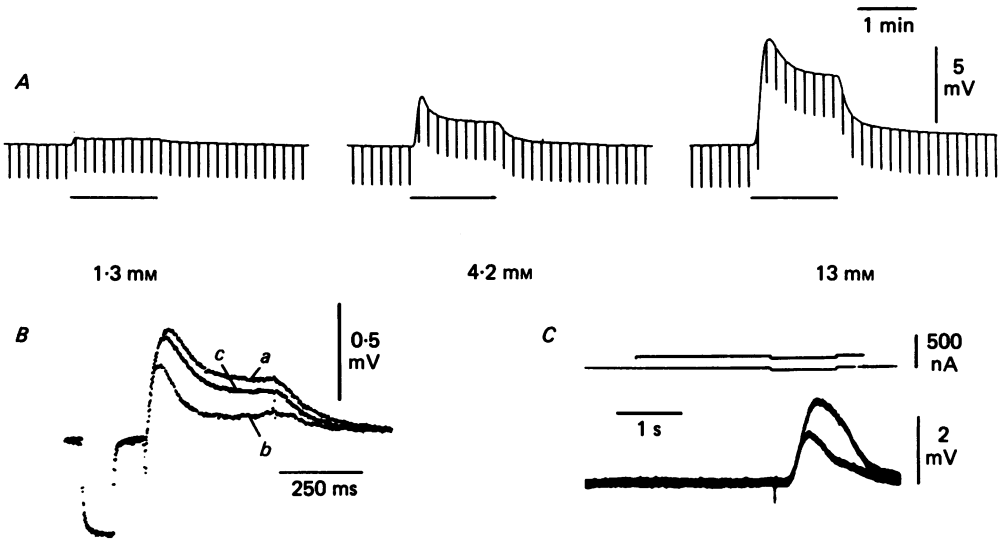

Fig. 2. *A*, responses of the same muscle fibre to bath application (bar) of 100 μM-glutamate in solutions containing 1·3, 4·2 and 13 mM-Ca^{2+}. Input resistance monitored by a 50 nA hyperpolarizing current pulse. *B*, superimposed responses to a 400 ms glutamate ionophoretic pulse recorded successively in the presence of 13 mM-(*a*), 3·25 mM-(*b*) and 13 mM-(*c*) Ca^{2+}. Each trace is the average of twenty responses elicited at 10 s intervals. Injections were stopped during 5 min following changes in the Ca^{2+} concentration of the perfusion solution. Input resistance was monitored by a constant current hyperpolarizing pulse preceding glutamate ionophoresis. *C*, effect of an ionophoretic pulse of Ca^{2+} on the response to a 1 s ionophoresis of glutamate. Two responses were elicited 30 s apart in a solution containing 3·25 mM-Ca^{2+} and were superimposed. The larger response was obtained when local Ca^{2+} concentration was increased by a 200 nA Ca^{2+} pulse starting 2 s before glutamate ionophoresis. Upper trace is current monitor.

Similar observations were made on preparations bathed in low Ca^{2+} solution when external Ca^{2+} concentration was varied only around the glutamate-sensitive spots by a local Ca^{2+} ionophoresis starting one or a few seconds before agonist application (Fig. 2*C*). Increasing the external Ca^{2+} concentration in this way resulted again in an enhanced response characterized by a longer time-to-peak and a slower decrease while the initial slope was not modified.

Kinetics of desensitization in normal and low Ca^{2+} solutions

The effects reported above strongly suggest that the decreased response recorded in low Ca^{2+} solutions could be due to a faster receptor desensitization. This was further demonstrated directly by using responses to brief glutamate pulses as test responses. The time course of onset of and recovery from desensitization were more precisely examined in experiments in which desensitization was induced by a long ionophoretic pulse of low intensity applied to a second high resistance glutamate pipette adjusted close to the same spot (Fig. 3) according to Katz & Thesleff (1957). Desensitization developed rapidly for very low ionophoretic currents. In the experiment shown in Fig. 3, the current applied to the desensitizing pipette was switched from +1 nA

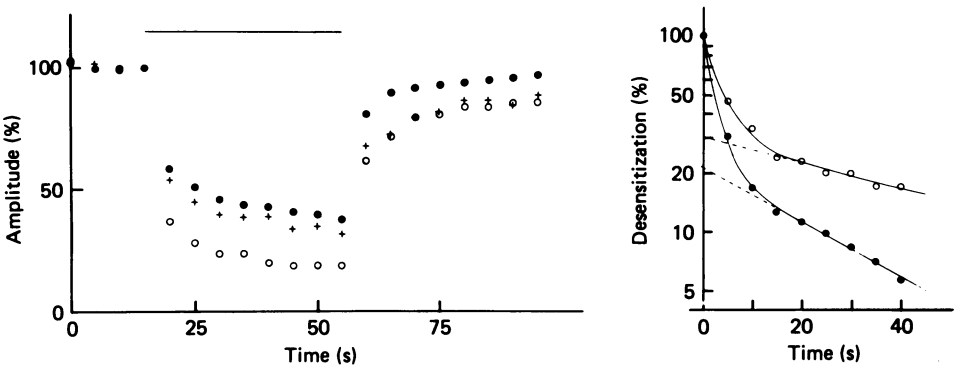

Fig. 3. Time course of desensitization and recovery in the presence of normal and low Ca^{2+} concentrations. Left: amplitude of a test response to a 5 ms, 400 nA glutamate pulse elicited every 5 s before, during and after a desensitizing glutamate pulse (bar) applied through a second pipette. The procedure was repeated twice in the presence of 13 mM-Ca^{2+}, then twice in the presence of 2·6 mM-Ca^{2+} and twice again after returning to 13 mM-Ca^{2+}. The responses recorded either in normal (●) or low Ca^{2+} (○) were averaged. The desensitizing pulse (1 nA, 40 s) started 200 ms after the fourth test pulse. Before returning to the normal solution, the effect of a weaker (0·5 nA) desensitizing pulse was also examined in the low Ca^{2+} solution (+). The amplitude of the fourth test response, taken as 100%, was 1·2 mV in normal solution and 0·9 mV in low Ca^{2+} solution. Right: semilogarithmic plot of the phase of recovery. The dashed line was determined by linear regression from the last six points.

(braking current) to −1 nA, a situation which could mimic leakage from a poorly controlled low resistance pipette. Glutamate thus applied did not induce any detectable change in membrane potential. Again, it was found that the test response declined faster and recovered more slowly when external Ca^{2+} was decreased. The higher proportion of desensitized receptors at the end of the desensitizing pulse in low Ca^{2+} solution could not account for the slower recovery: when the desensitizing glutamate current was reduced from 1 to 0·5 nA, the time course of desensitization in low Ca^{2+} was roughly comparable to that observed in normal Ca^{2+} using a 1 nA desensitizing glutamate current (Fig. 3). However, the time course of recovery was similar to that observed in low Ca^{2+} following the 1 nA desensitizing pulse.

Whatever the Ca^{2+} concentration, both onset of and recovery from desensitization occurred first rapidly, then more slowly. Due to uncertainties concerning the time

needed for equilibration of the glutamate concentration at the sensitive spot and due to the fact that the steady state of desensitization took a long time to reach, no quantitative analysis of the time course of onset was done. Such an analysis was easier for the phase of recovery which was, when plotted in semilogarithmic coordinates, well fitted by the sum of two exponentials. This is shown in the right part of Fig. 3 for the experiment reported above. In this case, decreasing Ca^{2+} concentration from 13 to 2·6 mM increased the time constant of the fast and slow components (2·5–3·8 s and 31–67 s respectively) and also increased the proportion of slowly recovering glutamate-activated channels from 20 to 30%. This latter proportion appeared, in other experiments, to be most affected by the duration of the desensitizing glutamate pulse. No slow components could be clearly detected, even in the presence of 2·6 mM-Ca^{2+}, when the desensitizing pulse was shorter than 10 s.

Effect of ionophoretically applied Ca^{2+} on desensitized receptors

As shown in Fig. 2*C*, the time course of the glutamate response can be modulated by a local Ca^{2+} ionophoresis preceding the response by a few seconds as well as by changing the Ca^{2+} concentration of the perfusion solution. Though this strongly suggests that Ca^{2+} acts at an ectocellular site of the receptor, it cannot be excluded that the Ca^{2+} action results from its increased participation in the cation influx triggered by agonist binding. This possibility could be discarded for the phase of recovery from desensitization (the kinetics of which depend, as shown just above, on the external Ca^{2+} concentration) by experiments such as that summarized in Fig. 4. The preparation was perfused in a low Ca^{2+} solution and two responses (*a* and *b*) were elicited at 1 s interval at the same sensitive spot. Intensity was adjusted so that the second response (*b*) was clearly decreased due to incomplete recovery from desensitization. When the local Ca^{2+} concentration was transiently increased between the two responses by Ca^{2+} ionophoresis, the second response (*d*) was then less decreased than when the Ca^{2+} pulse was omitted (*b*). A control response preceded by the same Ca^{2+} pulse in the absence of a desensitizing pulse was also recorded (*e*). A comparison of the different responses superimposed and displayed at a faster sweep speed (Fig. 4, bottom, *b* and *d*) shows that, after the Ca^{2+} pulse, the ringing phase of the second response was faster. This could not be due to the fact that, after Ca^{2+} ionophoresis, the Ca^{2+} concentration had not returned to the initial value when the second response was elicited. As shown above, Ca^{2+} affects the decaying phase of the response but not its initial slope. In the present case, additional evidence was given by the comparison of the responses obtained with or without Ca^{2+} ionophoresis in the absence of previous desensitizing glutamate application (*a*, *c* and *e*). In both situations, the slope of the rising phase was similar, a slightly longer time-to-peak characterizing the response elicited after the Ca^{2+} pulse (*e*). It could then be concluded that the transient increase in Ca^{2+} concentration increased the rate of recovery. Since only a very small number of glutamate-activated channels possibly remained open during the Ca^{2+} pulse, the cation effect could not result from an increase in intracellular Ca^{2+} concentration due to Ca^{2+} entry. Such experiments, together with failure to detect changes in the kinetics of desensitization and recovery following intracellular ionophoresis of either Ca^{2+} or EGTA, demonstrated that Ca^{2+} acted at an ectocellular site.

Ca^{2+} dependence of responses to brief glutamate pulses

In the experiments reported above, where responses to brief glutamate pulses were used as test responses for evaluating desensitization, lowering the external Ca^{2+} concentration led to an important decrease of the test response itself which could not be accounted for by the decrease in input resistance. For instance, in the experiment shown in Fig. 3, the amplitude of the test response in the presence of 2·6 mM-Ca^{2+} was only 75% of its value in the presence of 13 mM-Ca^{2+}. Since responses to brief

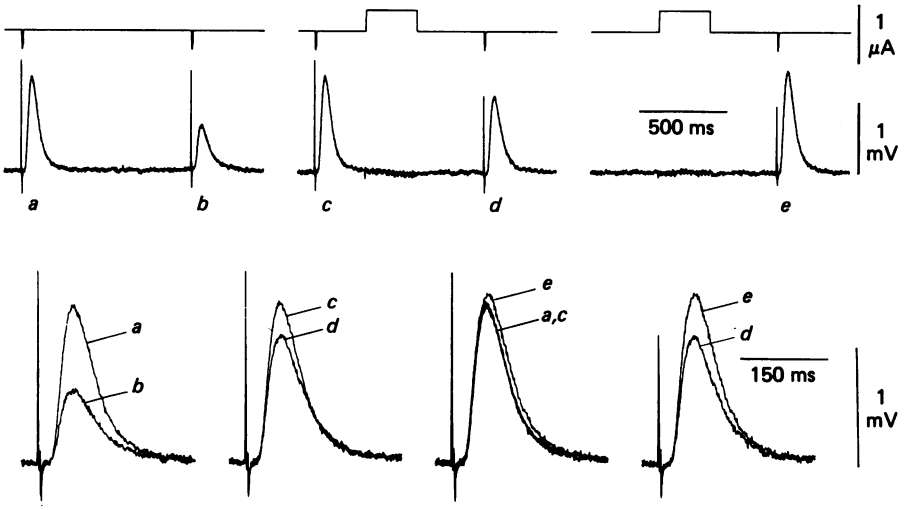

Fig. 4. Increase of the rate of recovery from desensitization by a transient increase in external Ca^{2+} concentration. Top: two responses (*a* and *b*) to a 5 ms, 325 nA glutamate pulse elicited 1 s apart in a solution containing 2·6 mM-Ca^{2+} (left). Responses (*c* and *d*) to the same glutamate applications when the Ca^{2+} concentration was transiently increased by a 300 ms 365 nA pulse starting 300 ms after the first glutamate pulse (middle). Response (*e*) to a single glutamate pulse preceded by the Ca^{2+} pulse in the absence of previous glutamate application (right). Each record is the average of five sweeps. Bottom: superposition of the different responses at a faster sweep speed.

agonist application were expected to be much less affected by desensitization than responses to longer pulses or bath application, it was necessary to determine if a faster desensitization could also account for the reduced response in low Ca^{2+} solutions in that case. The responses to glutamate pulses of 2–10 ms of various intensity were studied successively in the presence of either 13 or 2·6 mM-Ca^{2+}. The effect of the external Ca^{2+} concentration appeared clearly dependent on both duration and intensity of the glutamate pulse (Fig. 5). For the lowest agonist dose, the responses were quite similar in normal and low Ca^{2+} solutions. For higher doses, the responses in low Ca^{2+} were characterized by a smaller amplitude (Fig. 6*A*), shorter time-to-peak (Fig. 6*B*) and a faster decline (Fig. 6*C*). By contrast, the initial slope was unaffected (Fig. 5), indicating that only the activated glutamate receptor–channel complex was sensitive to external Ca^{2+}. Thus, as for longer agonist applications, a faster desensitization could account for the observed effects. Signs of desensitization would thus be detectable as soon as 10 ms from the start of the ionophoretic pulse for the highest intensity.

The Ca^{2+} dependence of Con A-treated fibres

Additional evidence for the mechanism proposed above was obtained by examining how Con A, a lectin which blocks desensitization of the glutamate response, affected its Ca^{2+} dependence. After recording responses to glutamate pulses of increasing intensity in normal and low Ca^{2+} solutions, responses were again recorded in normal Ca^{2+} solution in order to control their stability, then Con A was added to the perfusion

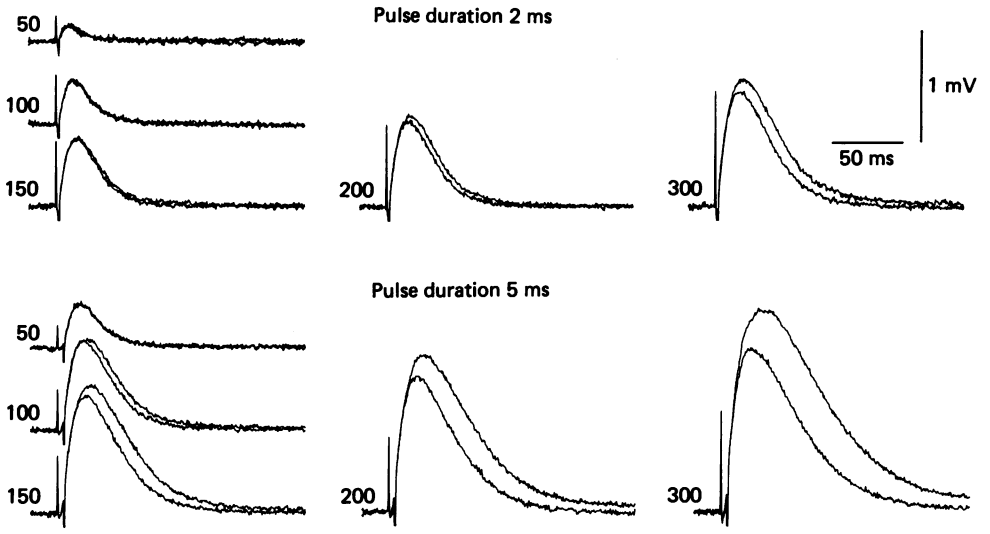

Fig. 5. Effect of lowering the external Ca^{2+} concentration on the response to brief glutamate ionophoretic pulses. Responses to 2 ms (top) or 5 ms (bottom) pulses of increasing intensity from 50 to 300 nA were applied to the same spot. They were recorded successively in the presence of 13 mM-Ca^{2+} and 2·6 mM-Ca^{2+} and were superimposed. When the two responses are not superimposable, the smaller and briefer one is that recorded in low Ca^{2+} solution. Numeric values preceding the responses refer to the pulse intensity. Each trace is the average of five responses as described in the legend of Fig. 6. Input resistance decreased by only 2% following Ca^{2+} reduction.

solution. The effect of the lectin was monitored by recording at intervals of 30 or 60 s a train of three responses in which, before Con A introduction, desensitization clearly occurred (*a* in Fig. 7 *B*). During the lectin action, apparent desensitization progressively subsided and changed to augmentation (Fig. 7 *A*). After Con A washing, no additional changes occurred and the responses to glutamate pulses of increasing intensity were again recorded in normal and low Ca^{2+} solutions (Fig. 8). When compared to those recorded before Con A treatment, the responses of the treated fibres were less affected by the reduction of external Ca^{2+}. For instance, in the case of the experiment shown, the responses of the Con A-treated fibre to a 5 ms pulse of 100 nA had now the same time course in the presence of either 13 or 2·6 mM-Ca^{2+}. For higher intensities, the differences in amplitude, time-to-peak and rate of decrease induced by lowering external Ca^{2+} were still present but clearly attenuated (Fig. 9). When the responses to glutamate pulses of several seconds were tested at that stage, signs of desensitization could however be detected and it was thus very likely that the incomplete blockade of Ca^{2+} dependence was related to the incomplete blockade of desensitization.

Treatments by higher doses of Con A (200–500 μg/ml) confirmed that the Ca^{2+} dependence could be irreversibly eliminated by the lectin. The responses to bath application were either unchanged or even potentiated when external Ca^{2+} was reduced from 13 to 1·3 mM (Fig. 10*A–C*). For the seven fibres in which the responses to 50–100 μM-glutamate were thus examined, the 10-fold decrease in external Ca^{2+} induced a mean increase of 15 % of the response amplitude. Because of the decreased input resistance in low Ca^{2+}, the potentiation is probably even higher. Con A itself

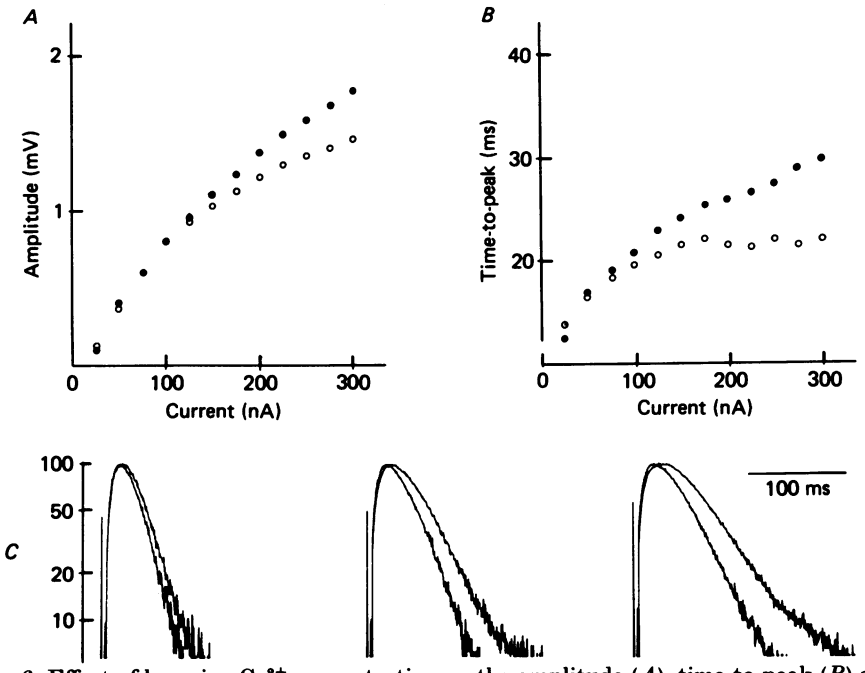

Fig. 6. Effect of lowering Ca^{2+} concentration on the amplitude (*A*), time-to-peak (*B*) and rate of decrease (*C*) of the response to a 5 ms glutamate pulse as a function of the pulse intensity. The responses to a series of five ionophoretic pulses, delivered at 5 s intervals were measured once a minute and averaged. The intensity of each set of pulses was increased by steps of 25 nA. The procedure was first applied in the presence of 13 mM-Ca^{2+} (●), then after 5 min washing with a solution containing 2·6 mM-Ca^{2+} (○). Samples of the responses are shown at the bottom of Fig. 5. The effect of external Ca^{2+} on the rate of decrease is shown in *C* where the responses to 100, 200 and 300 nA (left to right) are plotted in semilogarithmic coordinates. Peak value is taken as 100. The faster decrease corresponds to responses recorded in low Ca^{2+} solution.

neither modified input resistance nor prevented its decrease when the external Ca^{2+} concentration was lowered. It should be noted that repolarization of the fibres during the wash-out of glutamate was slower in low Ca^{2+} solutions than in normal ones (Fig. 10*A–C*).

The Con A treatment did not eliminate the ability of Ca^{2+} ions to flow through the glutamate-activated channels: in isotonic Ca^{2+} solutions, responses recorded from an untreated muscle and those simultaneously recorded from the symmetrical muscle of the same abdominal segment pre-treated by a high dose of Con A (500 μg/ml for 90 min) were quite similar (Fig. 11, right). Interestingly, no apparent desensitization was then detectable in the untreated preparation, whereas it was obvious for the

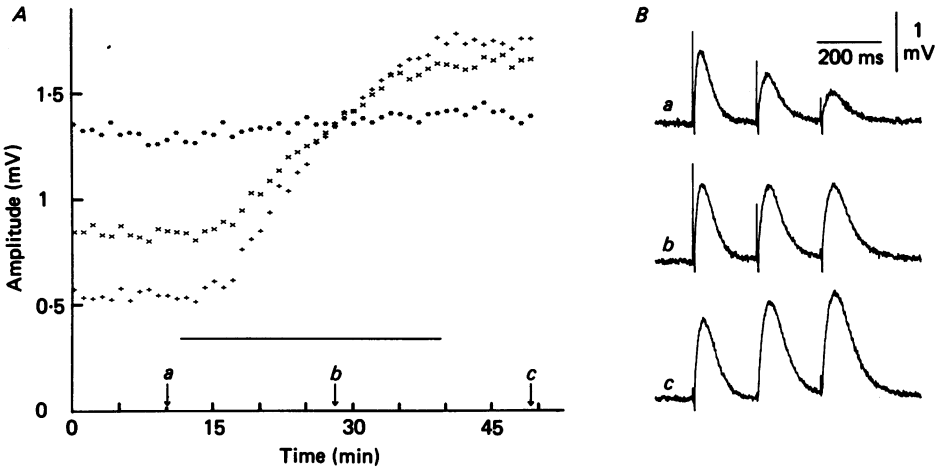

Fig. 7. Transformation of the glutamate response during blockade of desensitization by Con A. Three responses to a 5 ms 200 nA glutamate pulse were elicited at 200 ms intervals every minute. *A*, amplitude of the first (*), second (×) and third (+) responses is plotted *vs.* time. Con A (100 μg/ml) was applied for 28 min at the period indicated by the bar. *B*, samples of the responses recorded before (*a*), during (*b*) and after (*c*) Con A application as indicated by arrows in *A*. Same experiment as for Figs. 5 and 6.

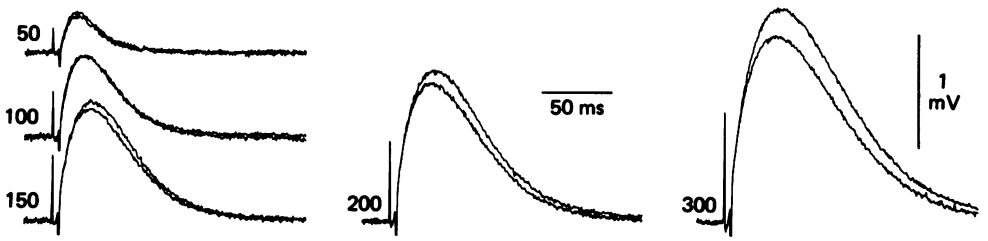

Fig. 8. Time course of responses to a 5 ms glutamate pulse of increasing intensity in the presence of either 13 or 2·6 mM-Ca^{2+} after a 28 min exposure to 100 μg Con A/ml and washing. The control responses recorded before Con A application are shown at the bottom of Fig. 5. Above 100 nA, the smaller response of each pair is that recorded in low Ca^{2+} solution.

control response recorded in normal solution before replacement of NaCl by an osmotically equivalent amount of $CaCl_2$ (Fig. 11, left).

Co^{2+}, which like other divalent cations blocks the glutamate response, still depressed the response of Con A-treated fibres (50 % reduction by 10 mM-Co^{2+}) without affecting its time course. In untreated fibres, La^{3+} at the concentration of 0·5 mM was shown to prevent the fading of the response to bath-applied glutamate in low Ca^{2+} solutions. In Con A-treated fibres, 0·5 mM-La^{3+} added to solutions containing 13 mM-Ca^{2+} reduced the glutamate response by 10–20 % (Fig. 10*D*). In solutions containing 1·3 mM-Ca^{2+}, the responses (potentiated when compared to those recorded in the presence of 13 mM-Ca^{2+}) were reduced by about the same amount when either 0·5 mM-La^{3+} or 11·7 mM-Ca^{2+} were added. In a higher concentration (5 mM-La^{3+}), a stronger blockade was obtained (Fig. 10*E*).

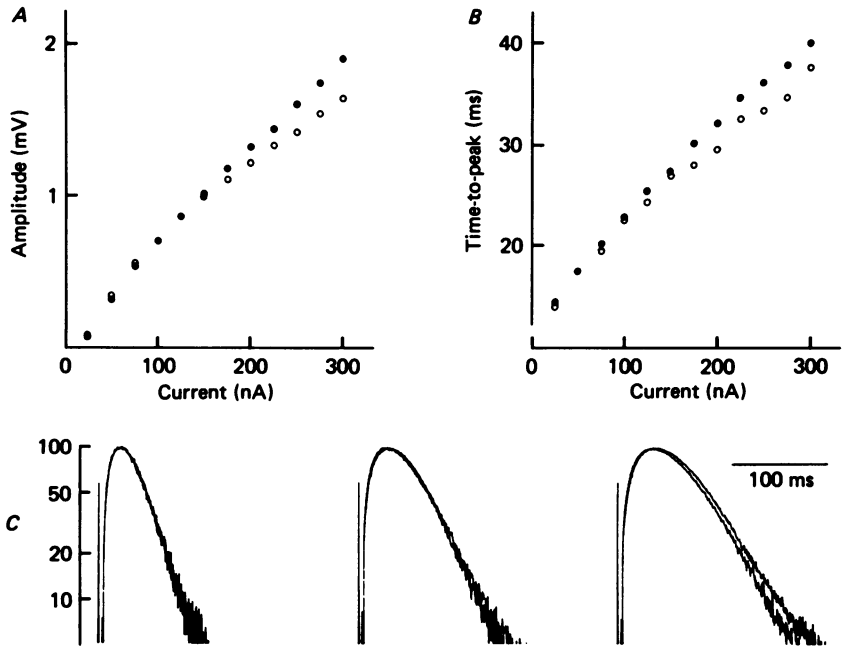

Fig. 9. Effect of lowering Ca^{2+} concentration on the amplitude (*A*), time-to-peak (*B*) and rate of decrease (*C*) after a 28 min exposure to 100 μg Con A/ml and washing. Compare with control results, obtained using the same protocol before Con A application, which are shown in Fig. 6.

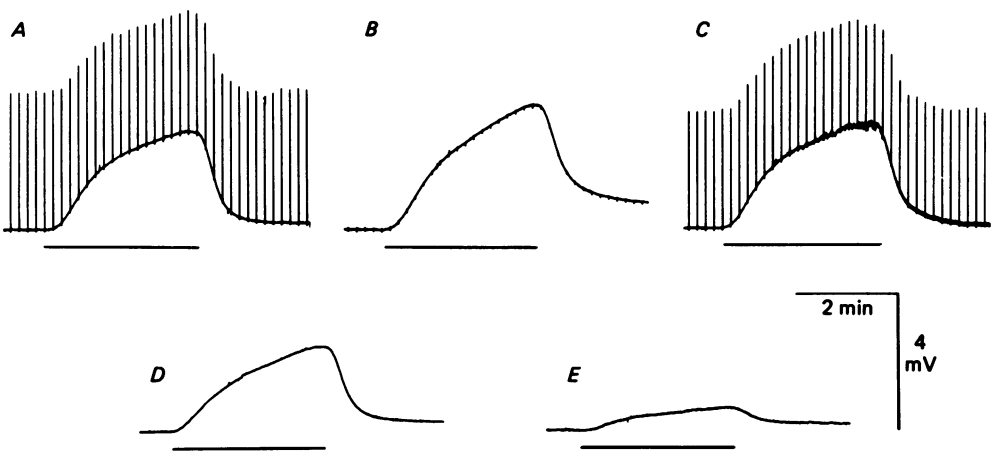

Fig. 10. Irreversible blockade of the Ca^{2+} dependence by Con A. Responses of the same fibres to 75 bath-applied μM-glutamate (bar) recorded in the presence of 13 mM-Ca^{2+} (*A*), 1·3 mM-Ca^{2+} (*B*) and after return to 13 mM-Ca^{2+} (*C*). Upward deflexions are post-synaptic potentials elicited by stimulation of an excitatory axon. The muscle had been treated 20 h before by 500 μg Con A/ml for 1 h then washed and stored at 4 °C in normal Ca^{2+} solution before use. Bottom: responses to the same glutamate application in a solution containing 13 mM-Ca^{2+} to which either 0·5 mM-La^{3+} (*D*) or 5 mM-La^{3+} (*E*) was added.

Modification of the glutamate response by Con A

In addition to its effect on the desensitization process, Con A was found to block the glutamate response partially. During treatment by the lectin, the initial slope of the response to brief (1–5 ms) glutamate pulses decreased. The phenomenon may easily escape attention since it is counteracted by the simultaneous elimination of desensitization often resulting in an unchanged response amplitude. For instance, in the experiment shown above (Fig. 7), amplitude of the response to the first pulse did not change whereas the rising slope decreased by about 15% (not shown). In other experiments, Con A clearly decreased the response amplitude. Two out of the seven experiments in which this was observed are shown in Fig. 12. A 40–50% reduction in the response amplitude occurred with a time constant in the order of 10 min. In Fig. 12*A*, the full blocking effect was obtained with a dose of 200 μg/ml, a further increase to 500 μg/ml having little effect. A similar blockade is shown in Fig. 12*B* with a dose of 400 μg/ml, whereas lower doses up to 100 μg/ml had no blocking effect on the amplitude, but slightly reduced the initial slope. However, addition of only 100 μg Con A/ml immediately affected the amplitude in other experiments. Due to this variability, it was not possible to relate the blocking effect to the dose of Con A. Presumably, there exists a great variability in the accessibility of the lectin to the sensitive spots. In one experiment, the blockade of desensitization, evaluated with repeated glutamate pulses (see Fig. 7), started within 1 min of the lectin application, indicating an easy access to the receptors. In another experiment, doses of up to 500 μg/ml applied for 65 min failed to block desensitization and did not affect the response amplitude.

DISCUSSION

The results reported above show that the Ca^{2+} dependence of the glutamate response can be explained by an action of the cation on the desensitization process. As expected in that case, the responses to brief pulses of low intensity were unaffected when the external Ca^{2+} concentration was reduced (Fig. 6). This definitely eliminates the possiblity that a part of the response could be mediated by transmitter released in a Ca^{2+}-dependent manner from the nerve terminal following activation of presynaptic receptors (Thieffry, Bruner & Personne, 1980). For longer or stronger pulses or for bath application, the smaller responses recorded in low Ca^{2+} solutions were characterized by a shorter time-to-peak and a faster decline, whereas their rising slope was not modified. Thus, Ca^{2+} does not affect the number of activatable receptors and acts only on the activated glutamate receptor–channel complex. The above results contrast with those of previous studies (Thieffry & Bruner, 1978; Nickell & Boyarsky, 1980) where the reduction of external Ca^{2+} concentration was shown to block ionophoretic responses much more strongly than found in the present case. Such a discrepancy can be attributed to the better control of agonist leakage achieved in the present study by use of high resistance pipettes (see Dudel, 1975*a*, *b*). As shown in Figs. 1 and 3, the response is extremely sensitive to small leakage. The use of a less concentrated (0·1 M) glutamate solution also helped to minimize leakage, though adjustment of the braking current became more critical (Fig. 1).

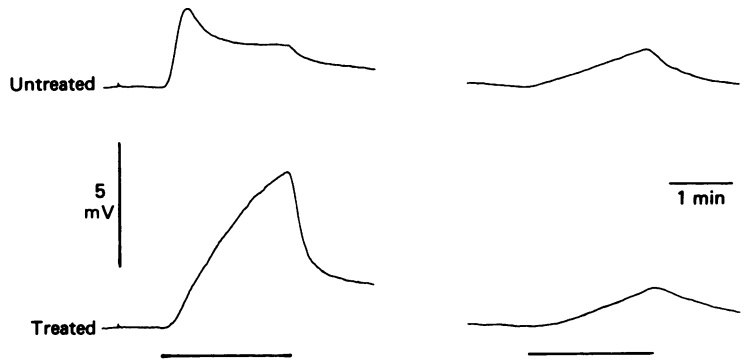

Fig. 11. Responses to bath-applied glutamate in normal solution (left) and in 'isotonic Ca^{2+}' solution (right). Two symmetrical muscles from the same abdominal segment were pinned side by side in the chamber after one of them had been treated for 90 min by 500 μg Con A/ml and washed. Responses to 100 μM-glutamate added for 2 min (bar) in the superfusion solution were simultaneously recorded from symmetrical sites of the two muscles.

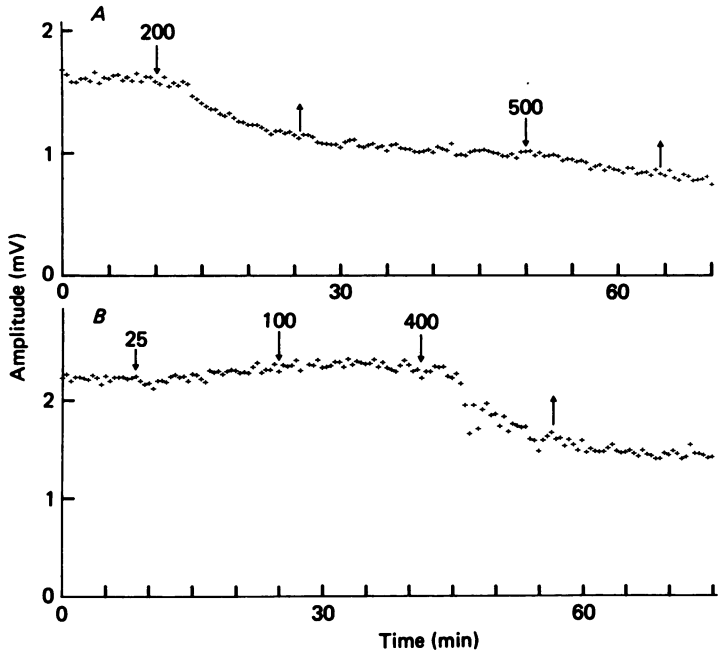

Fig. 12. Partial blockade of the response to brief glutamate pulses during Con A application. Downward arrows point to introduction of Con A at the concentration (μg/ml) indicated by the figures. Upward arrows point to washing. Two different experiments are shown in which glutamate pulse duration and intensity were respectively 2 ms, 300 nA (*A*) and 1 ms, 200 nA (*B*).

The more direct evidence for the Ca^{2+} dependence of the desensitization process arose from experiments such as that summarized in Fig. 3. These experiments demonstrated that Ca^{2+} affected the kinetics of both onset and recovery. In addition, they showed that these kinetics could not be described by a single exponential when agonist was present for more than a certain minimal time. The kinetics of recovery

were then well fitted by the sum of two exponentials. In this respect, the glutamate receptors appear to be similar to the cholinergic receptors of vertebrate neuromuscular junctions for which desensitization was recently shown to be a biphasic process (Feltz & Trautmann, 1982; Chesnut, 1983). For the latter preparations, fast and slow time constants of 11–12 s and 4–5 min respectively were reported. These values are larger than but not very different from those found for the glutamate receptors (2 and 30 s respectively in the presence of 13 mM-Ca^{2+}). Data concerning the kinetics of desensitization of glutamate receptors are much scarcer. For locust muscle receptors exposed to steady-state conditioning doses, desensitization was also reported to develop first at a fast rate then more slowly, but recovery was either exponential (time constant 1·27 s) or incomplete (Anis, Clark, Gration & Usherwood, 1981). Since the proportion of slowly recovering glutamate-activated channels was found here to be most affected by the duration of the desensitizing pulse, it may be that the protocol used in the study of locust muscle receptors did not permit detecting the slow component. To my knowledge, there are no published data that are directly comparable to those obtained in the present study concerning the kinetics of desensitization and recovery of crustacean muscle glutamate receptors.

The effect of Ca^{2+} on the desensitization of crayfish muscle glutamate receptors appeared, in the present study, to be quite different from that found for other receptors. At locust neuromuscular junctions, the post-junctional glutamate receptor desensitization is not significantly altered by changing the extracellular Ca^{2+} concentration (Clark, Gration & Usherwood, 1982). In crab muscles, the desensitization of a Cl^--mediated response activated by γ-aminobutyric acid is partially prevented by Ca^{2+} removal, but Ca^{2+} ions appear to be necessary for the process of recovery (Sarne, 1976). There is no general agreement about the involvement of Ca^{2+} in the desensitization of the vertebrate end-plate post-synaptic receptors (Anwyl & Naharashi, 1980). However, when a Ca^{2+} dependence was found, lowering the external Ca^{2+} concentration always caused a *decrease* in desensitization (Nastuk, 1977; Miledi, 1980). The results concerning the crayfish muscle receptors reported above thus do not coincide with any of the previous cases. From these examples restricted to motor synapses of vertebrates and arthropods, it is clear that the way in which Ca^{2+} may interfere with the desensitization process cannot be simply related either to the ionic form under which the agonist exists in the physiological solution or to the nature of the ions flowing through the channels opened by agonist binding.

Whereas desensitization of cholinergic receptors was reported to be affected by several divalent cations, more or less effectively (Magazanik & Vyskocil, 1970; Nastuk, 1977), the divalent cation dependence of desensitization of glutamate response appears to be much more specific for Ca^{2+}. None of the divalent ions (Mg^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+} or Co^{2+}) can overcome even partially the faster fading of the glutamate response in low Ca^{2+} solutions (Onodera & Takeuchi, 1976; Ashley & Campbell, 1978; Nickell & Boyarsky, 1980; Thieffry, 1982). This eliminates the possibility that Ca^{2+} simply acts by screening surface charges. Another striking difference in the ways by which Ca^{2+} affects the desensitization of acetylcholine- and glutamate-operated channels is its site of action. There is now good evidence that Ca^{2+} acts at an intracellular site in the case of motor cholinergic receptors (Miledi, 1980). On the contrary, the possibility of substituting Ca^{2+} by the impermeant cation

La^{3+} (Thieffry, 1982), and the results obtained in the present work using extracellular Ca^{2+} ionophoresis, are strong arguments in favour of an action at an ectocellular site of or close to the glutamate receptor. It could be tempting to postulate that it is at this site that diltiazem, a Ca^{2+} antagonist which decreases the rate of recovery of glutamate responses (Ishida & Shinozaki, 1980), exerts its effect. However, the recent finding that external or internal application of diltiazem also affects the acetylcholine response of frog muscles bathed in Ca^{2+}-free solution (Miledi & Parker, 1981) makes this hypothesis less attractive.

The blockade of the Ca^{2+} dependence by Con A, a lectin which irreversibly eliminates desensitization of the glutamate receptors (Mathers & Usherwood, 1976; Shinozaki & Ishida, 1978) constitutes a second line of evidence for the involvement of Ca^{2+} in the desensitization process. The similarity of the time courses of the elimination of the Ca^{2+} dependence of the response and the blockade of desensitization supports the idea that both effects are linked and do not result from different mechanisms. The effect of Ca^{2+} removal on the time-to-peak of the glutamate response in particular (Figs. 6–9) could be used for evaluating the efficacy of a treatment by Con A. In Con A-treated preparations in which the glutamate response no longer desensitizes, or more precisely in which no apparent desensitization is detectable (see Werman, 1976), lowering the Ca^{2+} concentration potentiated rather than depressed the response to bath-applied glutamate, whereas a decrease would be expected in view of the reduced response to single quanta (Dudel, 1981). Furthermore, after Con A, the effects of agonist removal by washing were then also noticeably slower. This may suggest that Ca^{2+} removal also reduces glutamate uptake. Due to the very high apparent co-operativity of the response (Dudel, 1975*b*; Dekin, 1983), a small increase in effective agonist concentration could account for the observed potentiation. In untreated preparations, accumulations of agonist due to reduced uptake, especially around the innermost receptors of the bundle of electrically coupled fibres, could also increase the rate of desensitization. However, such a mechanism cannot alone account for the faster desensitization in reduced Ca^{2+} solution since a strong Ca^{2+} dependence of the response to bath application was still observed at low temperature, i.e. when glutamate uptake was presumably inactivated at all Ca^{2+} concentrations.

In addition to its action on the desensitization process, Con A is known to act on channel properties. It eliminates the potential dependence of glutamate-operated channel lifetime in arthropod muscle (Dudel, 1979; Mathers, 1981; Stettmeier, Finger & Dudel, 1983). In molluscan neurones, it reveals the presence of a glutamate-induced permeability change that is not detectable in the same untreated cell (Kehoe, 1978). In view of these data, the possibility was raised in a preliminary report (Thieffry, 1982) that Con A might block the Ca^{2+} dependence by eliminating the permeation of Ca^{2+} ions while increasing that of others. This possibility must now be discarded. On the one hand, Dekin (1983) clearly demonstrated that Ca^{2+} was about half as permeant as Na^{+} through the glutamate-operated channel of another crayfish muscle treated by Con A. On the other hand, similar responses were recorded from either treated or untreated muscles in isotonic Ca^{2+} solutions (Fig. 11).

Con A was also found to partially block the response to brief glutamate pulses. An increase in, rather than a depression of the responses, was observed in other preparations following Con A action (Shinozaki & Ishida, 1979; Dekin, 1983). In fact,

the potentiation of the response due to elimination of desensitization and increase in the channel lifetime may mask a simultaneous partial blockade. This discrepancy, which might simply reflect the conditions of glutamate ionophoresis, will have to be examined further.

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