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DISTRIBUTION AND PHARMACOLOGICAL PROPERTIES OF SYNAPTIC AND EXTRASYNAPTIC GLUTAMATE RECEPTORS ON CRAYFISH MUSCLE

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SUMMARY

1. The distribution of glutamate sensitivity was measured in the opener muscle in the walking legs of the crayfish (*Cambarus clarkii*). L-Glutamate was ionophoretically applied under visual control.

2. Bundles of a few muscle fibres were isolated and viewed with Nomarski optics. Two axons, presumably excitatory and inhibitory, branched widely over the surface of individual muscle fibres, forming numerous clusters of boutons or varicosities.

3. Glutamate sensitivity was measured from the slope of the dose–response curves obtained by ionophoretic application of L-glutamate and expressed as mV/nC. The highest sensitivities were about 100 mV/nC, obtained at the edge of synaptic boutons. The sensitivity declined to less than 5 % about 2 μm away from the synaptic terminal. The time course of glutamate potentials was approximately the same as that of spontaneous synaptic potentials.

4. Glutamate depolarization started within 300 μsec after ionophoretic release of glutamate. This time lag was shorter than the synaptic delay of the nerve-evoked synaptic potential measured with an extracellular micro-electrode. This indicates that glutamate depolarization results from a direct action on the post-synaptic receptor.

5. Application of L-α-kainic acid decreased the amplitude of the glutamate potential produced at the synaptic region, whereas kainate increased the amplitude of the glutamate potential at the extrasynaptic region. It is suggested that the pharmacological properties of the extrasynaptic receptor differ from those of the synaptic receptor. Possible mechanisms for the different actions of kainate are discussed.

INTRODUCTION

It has been observed that ionophoretic application of L-glutamate produces depolarization of the excitatory synaptic region of the crayfish muscle (Takeuchi & Takeuchi, 1964). This observation suggests that glutamate receptors are localized at the synaptic membrane. However, nerve terminals are distributed diffusely over the surface of the muscle fibre and a more refined technique is necessary to determine the precise distribution and characteristics of the receptors. There are several reports which indicate different effects of drugs on neurally evoked excitatory postsynaptic potential (e.p.s.p.) and externally applied glutamate response (e.g. Shinozaki &

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Shibuya, 1974; Shinozaki & Ishida, 1979). This discrepancy has been attributed to the presence of extrasynaptic receptors (Takeuchi & Onodera, 1975; cf. Miledi, 1960 for acetylcholine receptors). Therefore, it seems important to answer the following questions. (*a*) Are there extrasynaptic receptors? (*b*) If so, are their properties the same as those of synaptic receptors?

In the present experiments the synaptic region was visually identified with Nomarski optics and the distribution of glutamate sensitivity was measured by ionophoretic application. This method has been applied successfully to synapses on ganglion cells and on vertebrate neuromuscular junctions (McMahan & Kuffler, 1971; Peper & McMahan, 1972; Kuffler & Yoshikami, 1975). Glutamate receptors were found to be concentrated at the subsynaptic membrane. In addition glutamate receptors which have slightly different pharmacological properties extend to the extrasynaptic region.

METHODS

The experiments were done on the opener muscle of the dactyl in the first or second walking leg of the crayfish (*Cambarus clarkii*). A bundle of a few muscle fibres was isolated together with a part of shell and the central tendon attached and mounted in a small chamber. The bath chamber consisted of a cover glass 0·17 mm thick on which a 0·75 mm thick sheet of plastic magnetic material was glued. Over a 10 mm diameter circular area the plastic material was removed so that the cover glass was exposed. The walls of the chamber were made with a water repellent plastic material (White Dental Manufacturing, Philadelphia). The fine muscle bundle was stretched and attached to the magnetic sheet with fine needles which penetrated the shell and tendon of the muscle fibres near their insertions. The solution in the bath chamber had a volume of about 0·1 ml and the bath solution was exchanged continuously at a rate of 0·08 ml./min. The bathing solution was a modified Van Harreveld solution (mM): NaCl, 207·5, KCl, 5·4; $CaCl_2$, 18·8 and pH was adjusted to 7·2 by Tris maleate buffer. Experiments were done at 18 °C.

The live muscle fibres were viewed with Nomarski optics (Zeiss), usually with a 40× water-immersion objective whose metallic portion, in contact with the bathing medium, was coated with Teflon to prevent possible toxic effects. The working distance between objective and the surface of muscle fibres was about 1·5 mm. For more detailed observation a 100× oil-immersion lens was also useful. Such an objective, however, had too short a working distance to permit electrical recording. When the muscle fibres were too thick, the resolution of Nomarski optics was reduced. Therefore, relatively small animals with thin muscle fibres were selected. Occasionally small animals of about 20 mm body length were also used. The muscle fibres were usually treated with collegenase solution (60 u/ml, Type III, Sigma) for about 30 min before mounting in the bath chamber. While this procedure increased the visibility of the nerve terminal, the proteolytic activity of the enzyme was progressive and led to a deterioration of the preparation in about 1–2 hr. Under this condition the glutamate sensitivity, particularly that at the extrasynaptic region, was decreased.

For better observation of nerve terminals methylene-blue (about 0·5 mg/ml.) was occasionally added for a few min before mounting the preparation in the bath chamber. This procedure stained some of the terminals in about 10–30 min. The colouring usually faded in a further 15 min, at a time when spontaneous miniature potentials tended to appear over a period of about 10–30 min. The glutamate sensitivities were measured after methylene-blue staining (Plate 2*A* and *B*).

Recording micro-electrodes were filled with 3 M-KCl and potentials were registered differentially. Glutamate electrodes were filled with 1 M-Na-L-glutamate (pH 8·0). The electrodes had resistances of 150–300 MΩ. The ionophoretic currents were measured with an A-V converter between the bath and ground. A 1000 MΩ resistor was inserted in series with the glutamate electrode. The level of the braking current was very critical for the measurement of the sensitivity. The standard procedure was to position the glutamate electrode at the synaptic region under visual control with a relatively high braking current of about 5 nA. Pulses of injection current were applied every 10 sec and the braking current was reduced until the largest glutamate

potential was obtained without a sign of desensitization. The braking current was then increased by 1–2 nA to prevent small steady leakage of glutamate. Braking currents which were too large delayed the onset of glutamate potentials. Therefore, for our most precise measurements of the time course and latent periods of glutamate potential, the braking current was kept near the critical level. The braking current used was usually 3–5 nA.

For ionophoretic injection of kainate the micro-electrode was filled with 0·5 M-Na L-α-kainate (pH 8·0, Nakarai). Kainate and L-glutamate were applied either from separate barrels of double-

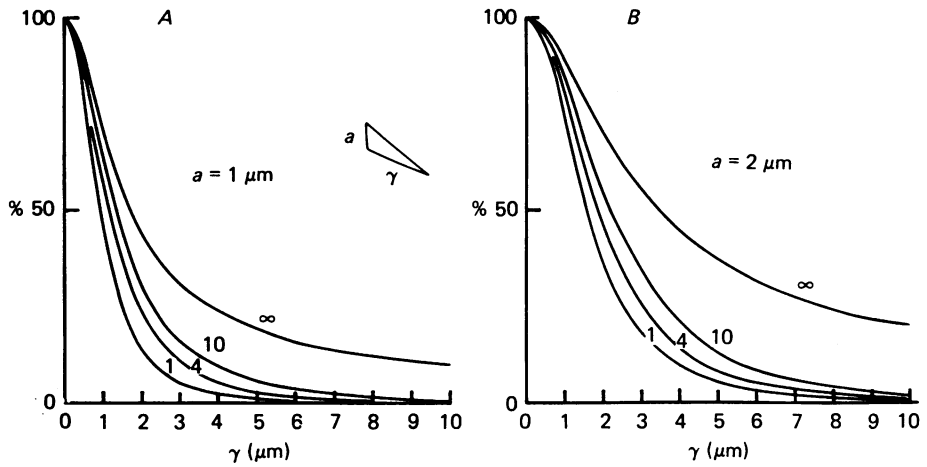

Fig. 1. Distribution of the maximal concentration of ionophoretically applied glutamate. Concentration was calculated from eqns. (1) and (2). Ordinates, the maximal concentrations at various points on the surface relative to that at the centre. Abscissa, distance on the surface. Distance between the tip of electrode and the surface is taken as 1 μm (A) and 2 μm (*B*), respectively. Numbers attached indicate the duration of the injection (msec).

barrelled micro-electrodes, or from separate micro-electrodes. Since kainate produced no potential change, a relatively high level of braking current was adopted to prevent leakage. Micro-electrodes were positioned with three dimensional oil-driven micro-manipulators (Narishige). The accuracy of the manipulator was sufficient for the present purpose and the manipulator was small enough to assemble several manipulators around the preparation.

For the present experiment, it is necessary to apply glutamate to a localized area on the muscle surface. The diffusion of glutamate is calculated from the diffusion equation (Takeuchi & Onodera, 1975; Onodera & Takeuchi, 1976). When a substance is applied at a rate of q for the period t', from a point source facing an infinite plane which is impermeable to the substance, the concentration of the substance (C) at a given point on the plane is expressed as

$$C = \frac{q}{2\pi D\sqrt{(a^2+r^2)}} \operatorname{erfc}\left(\frac{\sqrt{(a^2+r^2)}}{2\sqrt{(Dt)}}\right) \quad (t \leqslant t') \tag{1}$$

$$= \frac{q}{2\pi D\sqrt{(a^2+r^2)}}\left\{\operatorname{erfc}\left(\frac{\sqrt{(a^2+r^2)}}{2\sqrt{(Dt)}}\right) - \operatorname{erfc}\left(\frac{\sqrt{(a^2+r^2)}}{2\sqrt{D(t-t')}}\right)\right\} \quad (t \geqslant t'), \tag{2}$$

where t is the time after the start of the application; D, the diffusion constant; a is the distance between the point source and the plane and r, the distance on the plane; and erfc (Z) stands for

$$\operatorname{erfc}(Z) = 1 - \frac{2}{\sqrt{\pi}} \int_0^Z e^{-u^2}\, du \tag{3}$$

The relative value of the peak concentration at various distances from the centre is shown in Fig. 1, assuming that the diffusion constant is similar to that of glutamine ($7{\cdot}62 \times 10^{-6}$ cm^2/sec at 25 °C; Longsworth, 1953). The amount of glutamate that reaches the muscle surface by diffusion depends critically on the distance between the tip of the electrode and the muscle

surface (a) and on the pulse duration (t'). Therefore, the glutamate electrode was brought as close as possible to the muscle surface, until the current artefact appeared on the potential recording. The position of the electrode tip was photographed and its distance from the edge of bouton on the muscle surface was measured on the photomicrograph. The pulse duration used was usually 0·5–4 msec. In order to determine the lower sensitivities of the extrasynaptic region 10 msec pulses were also used.

If the distance between the electrode tip and the muscle surface was 1 μm, the peak concentration of glutamate 5 μm away from the centre would be 3% of the concentration at the center and less than 1% at 8 μm, when glutamate was applied with the 4 msec injection pulse. With the 10 msec pulse the concentration at 5 μm would be 5% and less than 1% at 10 μm.

RESULTS

Innervation of muscle fibre

The large nerve trunks often approach the muscle fibre almost at right angles and give off finer branches which run along the muscle fibre surface where they branch still further. Plate 1A shows a methylene-blue stained preparation. Two nerve axons, probably excitatory and inhibitory, ran almost parallel (Van Harreveld, 1939) and show, at periodic intervals, small swellings which appear as dark spots. These usually correspond to the synaptic regions. Nerve terminals were predominantly stained by methylene–blue, but other structures may also be stained, because the coloured terminal spots tended to become larger and sometimes fused during the progress of the staining.

Part B of Pl. 1 shows a terminal region, of a different muscle fibre, at higher magnification with a 100× oil-immersion objective after treatment with collagenase. Two distinct nerve branches run parallel before terminating with a cluster of bouton-like structures. At slightly different focal depth additional groups of terminals became visible at both sides on the muscle surface.

In part C, treatment with collagenase was omitted, and thus the synaptic region was covered with fine strands of collagen, reducing somewhat the clarity of structural detail. Schwann cell nuclei were often found in the proximal portion of the nerve terminals. In the right side of the muscle fibre of C nerve branches form contacts with a cluster of bouton-like structures (partly obscured by a glutamate electrode). Terminal branches spread further to the left more diffusely over the muscle fibre, forming many boutons or varicosities along their course. These boutons are sensitive to L-glutamate (arrows in Pl. 1C) and sample records are shown in the bottom.

The sensitivity to L-glutamate was tested at the cluster of boutons (Pl. 2A) and on the terminal branches with distinct varicosities (Pl. 2B). In these preparations some of the terminals were stained with methylene–blue.

Two nerve axons are visible in Pl. 2A. Arrows in both A and B indicate the points where L-glutamate was applied ionophoretically. Dark arrows show the points where the glutamate sensitivity was 8–30 mV/nC in Pl. 2A. and 2–4 mV/nC in Pl. 2B. Open arrows show the points where the sensitivity was less than 0·7 mV/nC in A and 0·2 mV/nC in B. The highest sensitivity to L-glutamate was obtained at the boutons or varicosities. The pattern of innervation in the opener muscle of the crayfish seems similar but more complex than that observed previously from methylene–blue staining (Van Harreveld, 1939; see however Lang, Atwood & Morin, 1972).

Glutamate sensitivity at the synapse

The glutamate pipette was brought close to a synaptic bouton under visual control and L-glutamate was ionophoretically applied with a pulse of 0·5 msec duration. Fig. 2*B* shows glutamate potentials. The muscle fibre had been treated with collagenase, most of the nerve terminal had been removed and therefore spontaneous

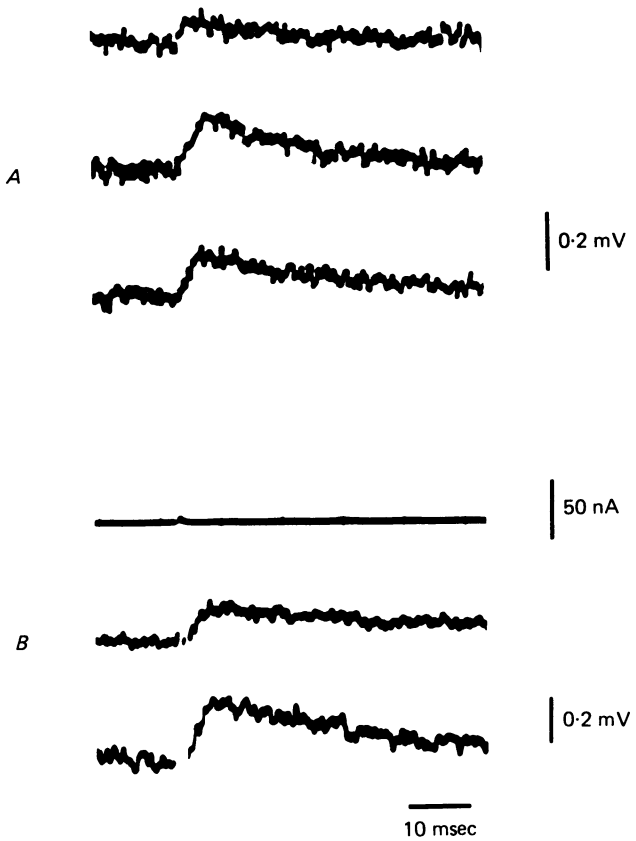

Fig. 2. *A*, spontaneous e.p.s.p.s recorded intracellularly from a muscle fibre. Three samples of the fastest time course are selected among those recorded from the muscle fibre. *B*, glutamate potentials produced in another muscle fibre of the same bundle as in *A*. The muscle fibre was treated with collagenase.

excitatory postsynaptic potentials (e.p.s.p.) were not recorded. The glutamate potential rose to its peak in about 3 msec. Spontaneous e.p.s.p.s were recorded intracellularly from another muscle fibre of the same muscle bundle which still had nerve contacts. The time course of spontaneous e.p.s.p.s was variable even in the normal, untreated muscles (Onodera & Takeuchi, 1978) and spontaneous e.p.s.p.s shown in Fig. 2*A* were among the fastest that we have seen in this muscle fibre. We have no evidence whether collagenase has effects on the time course of e.p.s.p.s, but these e.p.s.p.s had a similar time course as the glutamate potentials. However,

the falling phase of the glutamate potential in Fig. 2*B* was slightly slower than that of spontaneous e.p.s.p.s. Since the nerve terminal of this preparation had been removed, it is possible that the absence of glutamate uptake into terminals may account for the slower time course. Another possibility may be a protracted leakage of glutamate from the pipette after the end of the injection current.

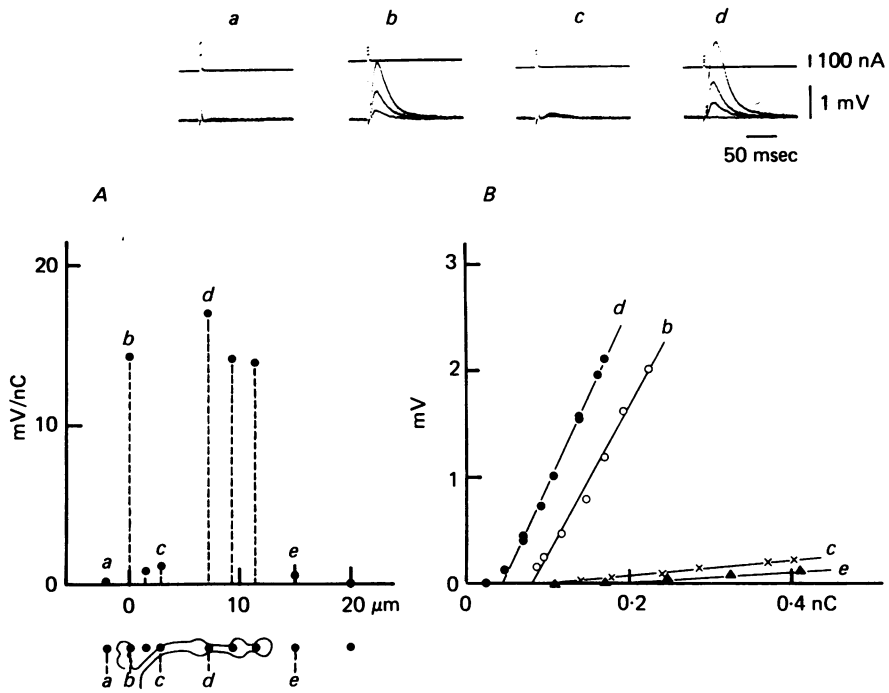

Fig. 3. *A*, glutamate sensitivity along a terminal branch, expressed as mV/nC. *B*, the sensitivity was measured from the slope of the dose–response curve. Sample records are shown on the top.

L-Glutamate was applied to various points along the nerve terminal and the dose was changed (Fig. 3). Each time the tip of the glutamate electrode was photographed and the position was measured from the microphotograph. The amplitude was plotted against the dose of glutamate (nC) and the sensitivity was measured from the linear part of the dose–response curve and expressed in mV/nC (Fig. 3*B*). The highest sensitivity was found at the edge of the boutons or varicosities and the sensitivity sharply decreased as L-glutamate was applied a very small distance away from the edge of the bouton. Fig. 3*A* shows that at about 2 μm from the edge of the bouton the sensitivity decreased to less than 5% of that at the very edge of the bouton. When L-glutamate was applied to the nerve axon between boutons, the sensitivity was also smaller than that at the edge of a bouton. These results suggest that glutamate receptors are localized mainly at the membrane under the bouton. E.p.s.p.s could be easily recorded with an extracellular microelectrode placed at the cluster of boutons. Therefore boutons may correspond to the excitatory synapse. However, when the sensitivity of each bouton within a cluster was tested,

some of the boutons were not sensitive to glutamate (see e.g. Pl. 2*A*). Since muscle fibres are supplied by two parallel running axons, one excitatory, the other inhibitory, some of the insensitive boutons may belong to the inhibitory terminals. Since the spatial discrimination of GABA sensitivity was not as good as that of glutamate sensitivity, it could not be decided whether glutamate insensitive boutons respond to GABA. However, when L-glutamate and GABA were applied ionophoretically from a double barrelled micro-electrode, the sensitive area to L-glutamate and GABA

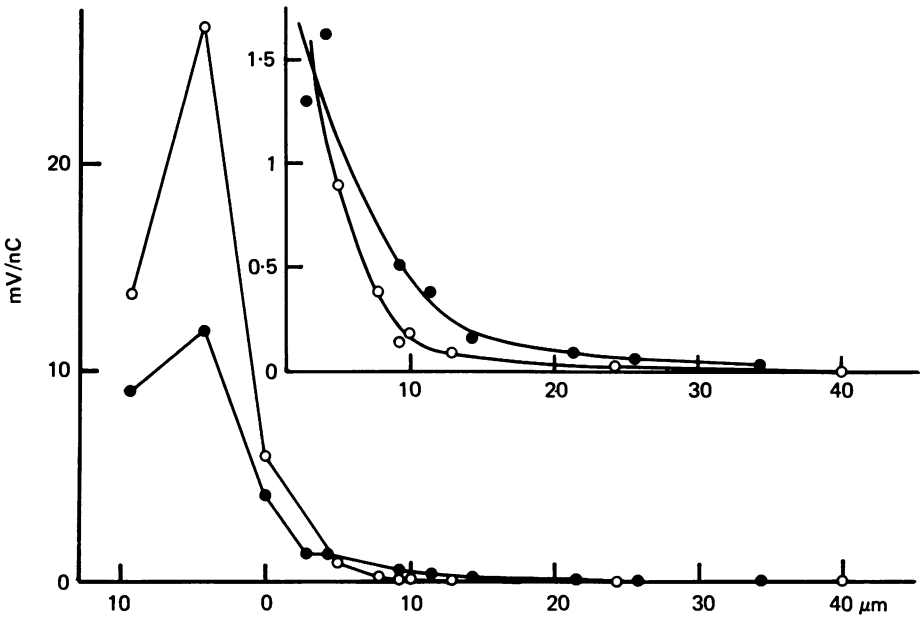

Fig. 4. Distribution of glutamate sensitivity. A cluster of boutons extended from 0 to negative on the abscissa over 15–20 μm in length. The origin corresponds to the right side edge of the cluster. Ordinate in the extrasynaptic region is expanded and shown in the inset. ○, the sensitivity recorded in the normal solution. ●, recorded in 0·2 mM-kainate.

almost overlapped each other, but occasionally it was found that in a cluster of boutons some portion was more sensitive to glutamate than to GABA and vice versa.

The glutamate sensitivity was usually 10–30 mV/nC and the highest sensitivity was 120 mV/nC. The sensitivity was several tens of times smaller than that of ACh-sensitive membrane in the vertebrate neuromuscular junction (Kuffler & Yoshikami, 1975). A relatively small value in sensitivity may be, at least partly, attributed to the low input impedance of the crayfish muscle. Mean value of the input resistance was 200 kΩ and the time constant 33 msec. Another possibility may be a relatively small synaptic area.

Since the glutamate sensitivity was remarkably low in the extrasynaptic regions, a more detailed distribution of extrasynaptic sensitivity was made (Fig. 4, open circles). A cluster of boutons extended over 15–20 μm and the right side edge of the cluster was taken as the origin of the abscissa. Two sample records within the cluster

are shown as hollow circles on the left of origin. The sensitivity sharply decreased at the extrasynaptic region but a small sensitivity extended to about 20–30 μm from the bouton. The ordinate at the extrasynaptic region is expanded and shown in the inset (open circles). Beyond 40 μm it was difficult to detect the sensitivity. The level of glutamate sensitivity at the extrasynaptic region was variable from preparation to preparation. It was also vulnerable to various conditions e.g. when the muscle was soaked in the collagenase solution for a long period of time, the glutamate sensitivity at the extrasynaptic region became even lower and the sensitivity was more critically localized to the bouton. Low sensitivity outside synaptic boutons may be attributed to the extrasynaptic receptors. But we cannot exclude the possibility that the low sensitivity was due to synaptic regions, buried in the muscle fibre, which were not detected by microscopy.

Contraction of the muscle fibre by glutamate

When L-glutamate was applied to the synaptic region, shortening of sarcomeres was observed under the microscope. The shortening was graded depending on the dose of glutamate applied. The contraction was not due to the depolarization of the membrane, because in many cases contraction was produced with the depolarization smaller than 1 mV. Furthermore, when Ca in the solution was replaced by Mg, the shortening was immediately stopped, before the amplitude of the glutamate potential was decreased in the Mg solution (Onodera & Takeuchi, 1976). It has been suggested that the synaptic membrane becomes permeable to Ca by the action of glutamate (Kusano, Miledi & Stinnakre, 1975; Onodera & Takeuchi, 1976) as observed for the ACh receptor (Takeuchi, 1963; Kuffler & Yoshikami, 1975). Therefore the graded contraction of the muscle fibre may be produced by an entry of Ca through the synaptic membrane. When spontaneous e.p.s.p.s appeared with high frequency, shortening of sarcomeres was also observed near the synaptic region. However, the magnitude of contraction produced by glutamate was very variable from preparation to preparation. It is not clear whether this difference is attributed to the different structure of the synapse or to the different membrane permeability.

Differential effect of kainate on the synaptic and extrasynaptic receptors

L-α-kainic acid which is structurally related to L-glutamate was used to analyse the pharmacological properties of the glutamate receptors. This drug increases the depolarization produced by bath-applied glutamate in the crayfish muscle. Yet it has little effect on the amplitude of the neurally evoked e.p.s.p.s (Shinozaki & Shibuya, 1974). It was suggested that kainate acts on the extrasynaptic receptors and thereby increases the glutamate response (Takeuchi & Onodera, 1975). Fig. 5*A* shows the glutamate potential recorded at the synaptic terminal and the ionophoretic application of kainate preceding the glutamate application reduced the amplitude of glutamate potential (Fig. 5*Ab*). Whereas, when L-glutamate was applied 8 μm away from the edge of the synaptic bouton, application of kainate increased the amplitude of the glutamate potential (Fig. 5*Bb*). Application of kainate itself produced no potential change.

The depression of glutamate potentials by kainate at the synaptic terminal was not due to the desensitization of the receptor because the glutamate potentials

remained at the same reduced level during the prolonged application of kainate and recovered to the control value immediately after the cessation of the kainate application (Fig. 5*Da*). Potentiation of glutamate potentials by kainate at the extrasynaptic regions also remained at a constant level during the application of kainate (Fig. 5*Db*).

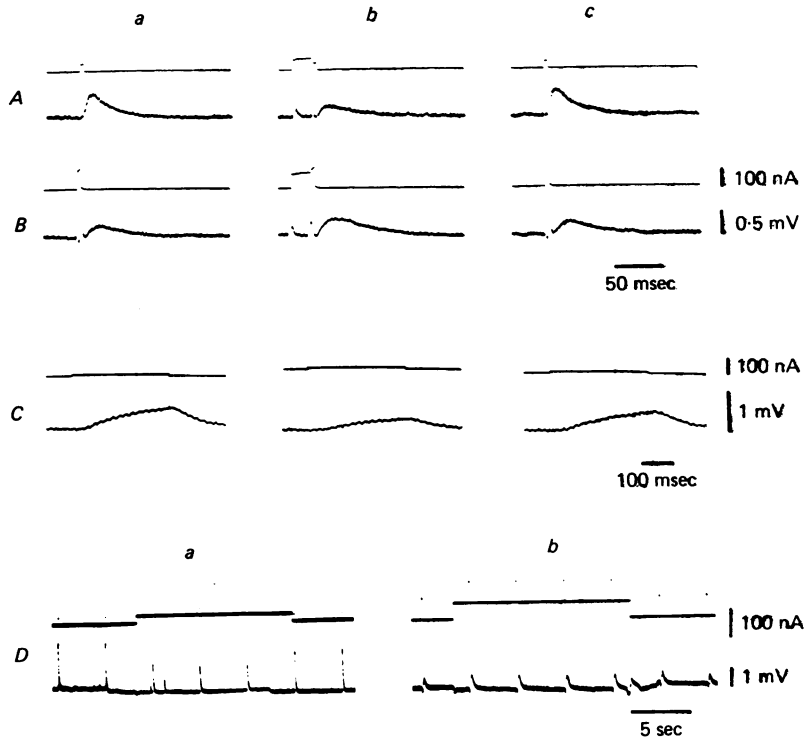

Fig. 5. Effect of kainate on the glutamate potential. L-Glutamate and L-α-kainate were applied ionophoretically and the injection currents were monitored on the upper traces. *A*, recorded at a synaptic bouton. *B*, recorded 8 μm away from the terminal. *C*, recorded at the synaptic bouton, but slow depolarization was produced by a small and prolonged application of glutamate. *a*, before, *b* simultaneous application of kainate and *c*, control after application of kainate. *D*, prolonged application of kainate, while short test pulses of glutamate were applied; *a*, recorded at the synapse and *b* at about 10 μm from the synapse.

Distribution of the glutamate sensitivity in a solution which contained 0·2 mM-kainate is shown as filled circles in Fig. 4. The glutamate sensitivity at the extrasynaptic region was increased by kainate, whereas it was decreased at the synaptic region.

These results suggest that the pharmacological properties of extrasynaptic receptors are different from those of synaptic receptors. However, glutamate diffuses over a considerable distance, when a large amount is injected (Fig. 1) and therefore possible errors due to diffusion must be considered.

Time course of the glutamate potential produced at the extrasynaptic region was relatively slow compared to that at the synaptic bouton (Fig. 5*B*). If the slow

depolarization is produced by the diffusion of glutamate to the synaptic receptor, it might be that the potentiating effect of kainate is a slow process and it enhances only a slow depolarization. This possibility was tested by applying a small and prolonged injection pulse through a glutamate electrode placed at the synaptic bouton. It was found that kainate depressed the slow depolarization as well as fast responses produced at the synaptic bouton (Fig. 5*C*).

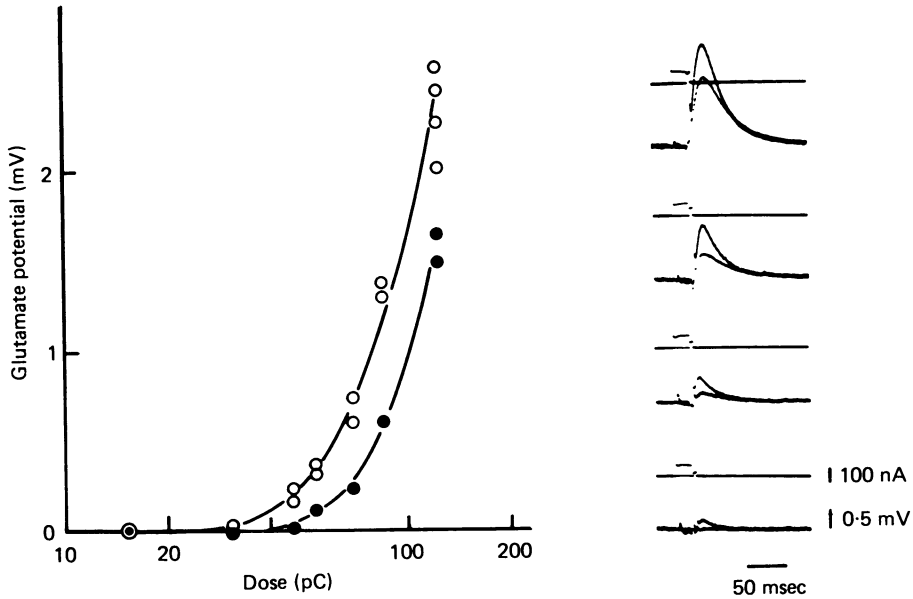

Fig. 6. Effect of kainate on the glutamate potential at a synaptic bouton. The dose of glutamate was changed. The glutamate potential (○) was depressed in the presence of kainate (●) at all doses tested. Sample records are shown on the right. Glutamate potentials with and without kainate are superimposed.

Another possibility may be that a small amount of glutamate diffused to the synaptic receptor and kainate enhanced the response produced by a low concentration of glutamate, while it depressed the response produced by a high concentration. A glutamate electrode was placed at the synaptic bouton and the dose of glutamate was changed while a constant dose of kainate was applied. As shown in Fig. 6 kainate depressed the glutamate response at all concentrations tested with little change in its time course.

In many cases, however, when the glutamate electrode was placed at the synaptic bouton and a large amount of glutamate was applied, the peak amplitude was depressed but the falling phase of glutamate potential was prolonged by kainate (Fig. 7*A a*). In this case some glutamate may have diffused to the extrasynaptic receptors whose response was enhanced by kainate.

At the extrasynaptic region (8 μm from the synapse) the small glutamate potential was enhanced with little change in the time course by application of kainate (Fig. 7*A b*). However, when the dose of glutamate was increased, kainate depressed the early part of the glutamate potential, but it enhanced the later part (Fig. 7*A c*). This result may be explained as follows: when a large dose of glutamate was applied, some

glutamate diffused to the synaptic receptor and produced depolarization which was depressed by kainate, while the depolarization of the extrasynaptic receptors was slow and it was enhanced by kainate.

Fig. 7*Ba* shows the dose–response relationship at the synaptic bouton. Open circles show the control and filled circles (1·2 nC) and crosses (2·0 nC) are the responses

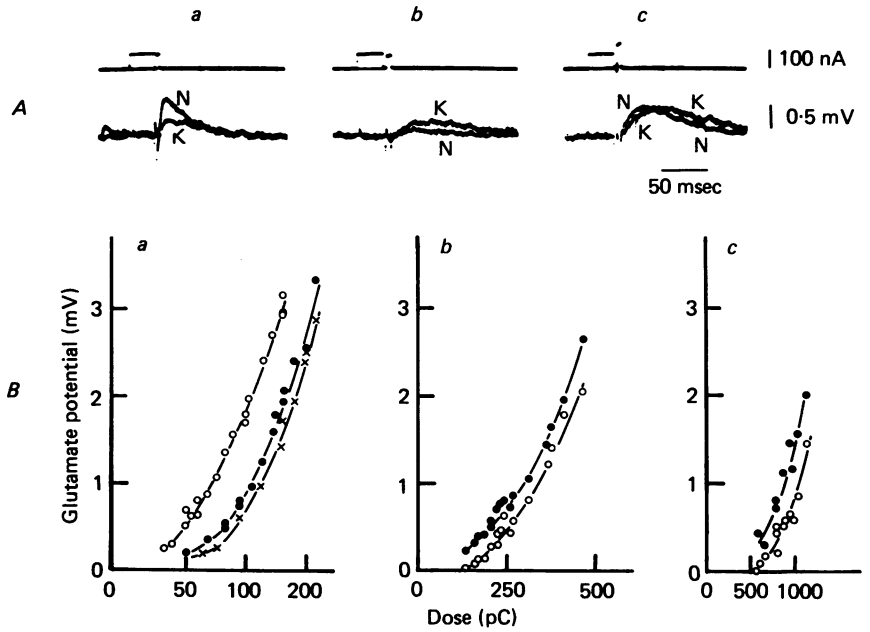

Fig. 7. *A*, effect of kainate on the time course of glutamate potential. Glutamate potentials without kainate (N) and with kainate (K) are superimposed. *a*, recorded at a synaptic bouton, *b* and *c*, recorded 8 μm away from the synaptic terminal. Dose of glutamate was increased in *c*. *B*, dose–response curve of glutamate potential. *a*, peak amplitude obtained at the synaptic bouton and plotted on log dose scale. ○, control. ●, with simultaneous application of kainate (1·2 nC). ×, with kainate (2·0 nC). *b*, amplitude of glutamate potential recorded at 8 μm away from the synaptic terminal and measured at fixed time of 40 msec after the start of the response, plotted on linear dose axis. ○, control and ● with kainate (1·2 nC). *c*, peak amplitude recorded 10 μm from the synaptic terminal and plotted on linear dose axis. ○, control and ● with kainate (1·2 nC).

during the ionophoretic application of kainate. The dose–response curves show an approximately parallel shift along the log dose axis. The result suggests that kainate is a competitive inhibitor of the synaptic glutamate receptor. Fig. 7*Bb* shows the dose–response relationship of the glutamate potential produced 8 μm from the terminal. The amplitude of glutamate potentials was measured at a fixed time (40 msec) after the start of the depolarization. Open circles are the control and filled circles are after application of kainate (1·2 nC). Depolarization was increased by kainate and the dose–response curve was shifted to the left on the linear dose axis. Another example is shown in Fig. 7*Bc* where glutamate was applied 10 μm from the synapse. The dose–response curve of peak amplitude showed an almost parallel shift along the linear dose axis.

Effect of kainate on the e.p.s.p.

If there are two types of glutamate receptors, it becomes important to determine the identity of the neurally activated receptor. In Fig. 8 successive samples of e.p.s.p.s are shown on the left and the averaged extracellular and intracellular e.p.s.p.s are shown on the right (128 single records). Kainate was ionophoretically applied in *B*. During the application of kainate the amplitude of extracellular e.p.s.p.s was

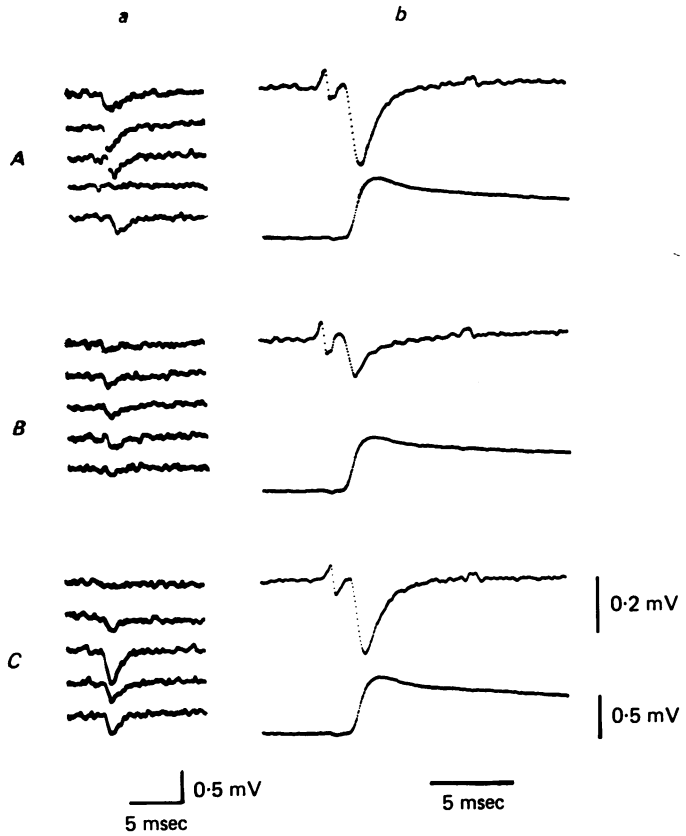

Fig. 8. Effect of kainate on the extracellular e.p.s.p. *a*, successive records of the extracellular e.p.s.p. *b*, averaged records of extracellular and intracellular e.p.s.p.s (128 single records). *A*, before *B* during ionophoretic application of kainate and *C* after.

decreased to about 50% of the control. Quantum content was $3{\cdot}4 \pm 0{\cdot}18$ (mean ± S.E., nine experiments) and $2{\cdot}8 \pm 0{\cdot}14$ (seven experiments) during the application of kainate. The amplitude and time course of the presynaptic nerve spike were not changed during the application of kainate.

Post-synaptic nature of glutamate action

It has been recently proposed that in the lobster muscle L-glutamate acts on the presynaptic nerve terminal to cause release of the endogenous transmitter which in turn produces the post-synaptic response (Colton & Freeman, 1975). This possibility

was tested by measuring the time couse of glutamate action. When the nerve-evoked e.p.s.p. was recorded with an extracellular micro-electrode, the presynaptic nerve spike and the e.p.s.p. were recorded. The synaptic delay was measured from the time between the peak of the presynaptic nerve spike and the start of the e.p.s.p. Fig. 9 shows the synaptic delay measured at two different temperatures which

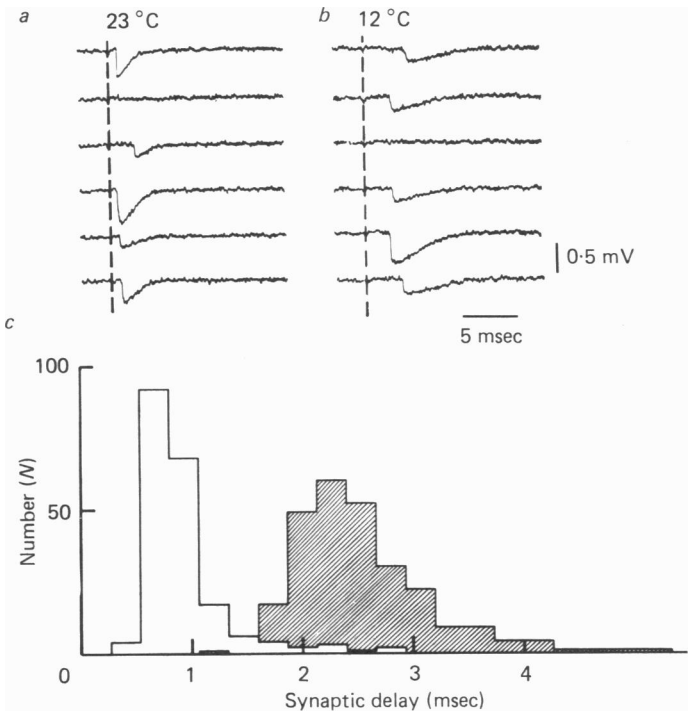

Fig. 9. Synaptic delay of the extracellular e.p.s.p. recorded at 23 and 12 °C. Histograms of synaptic delays at 23 and 12 °C are shown below.

showed a characteristic distribution as observed in the frog end-plate (Katz & Miledi, 1965*a*, *b*). At 23 °C the synaptic delay ranged between 0·4 and 2·9 msec and the peak value was 0·7 msec. At 12 °C the synaptic delay was between 1·2 and 5·3 msec with peak value of 2·3 msec (see also Bracho & Orkand, 1970). If L-glutamate were to release endogenous transmitter by depolarizing the presynaptic nerve terminal, the time delay between the application of glutamate and the start of depolarization should be longer than the synaptic delay.

To test this possibility the glutamate electrode was placed close to the synaptic terminal and a large dose of glutamate was applied. Fig. 10 shows the potential change produced by glutamate application with 4 msec injection pulse. After recording the glutamate depolarization, braking current was stopped and the glutamate receptor was desensitized. Application of glutamate produced only an artifact without glutamate depolarization. The artifact was subtracted from the glutamate depolarization and plotted in the lower part of Fig. 10. The glutamate depolarization increased almost linearly during the glutamate application. Since

the membrane time constant of the crayfish muscle is large, the linear depolarization would be expected when the membrane conductance was suddenly increased by a large amount of glutamate. When the linear part was extrapolated to the zero potential, it was found that the depolarization started 0·28 msec after the glutamate application. Since the present experiment was performed at 18 °C and the Q_{10} for

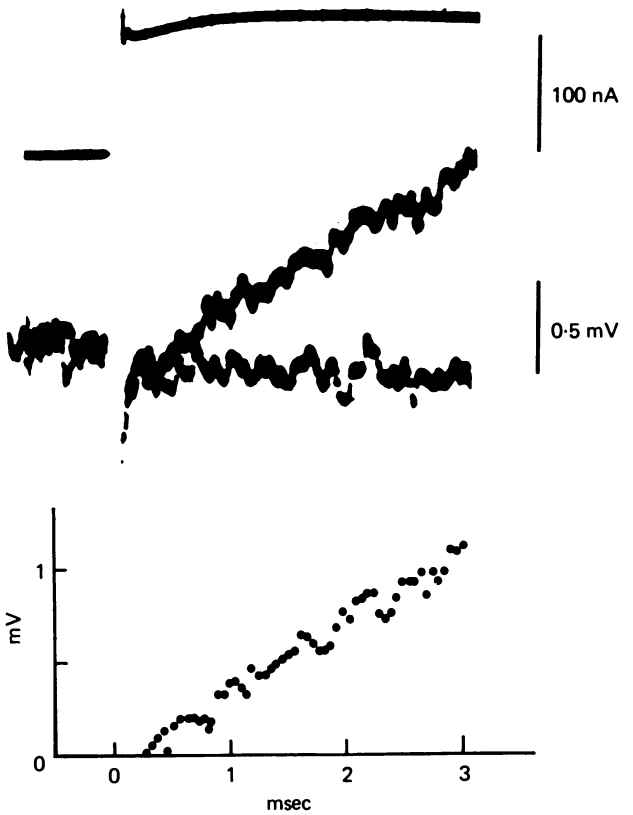

Fig. 10. Time course of the glutamate depolarization. L-Glutamate was applied with 4 msec injection pulse which is monitored on the top trace. Glutamate receptor was desensitized and artifact was superimposed on the glutamate depolarization. The artifact was subtracted from the glutamate depolarization and plotted on the lower graph.

the synaptic delay was 3, the minimum synaptic delay would be about 0·69 msec at 18 °C. These results are similar to those observed in the vertebrate neuromuscular junction (Katz & Miledi, 1965*a*) and they indicate that the post-synaptic processes of the glutamate action are fast compared to the synaptic delay.

The post-synaptic nature of the glutamate action was further supported by the observation that after removing the presynaptic nerve terminal, the post-synaptic membrane still responded to glutamate. When the muscle was soaked in relatively high concentration of collegnase (60 u/ml.) for more than one hour, most of the nerve terminal could be removed, although in many cases thin threads of a few μm in length remained. The glutamate sensitivity was compared at the synapses where the nerve terminal was removed and those where the nerve terminal was intact. The

sensitivity at the synaptic membrane was not changed by the action of collagenase. However, when the muscle was soaked in collagenase solution for a long time, the glutamate sensitive area was very critically localized to the synaptic region. We have no evidence so far but, collagenase may have removed some glutamate receptors, particularly in the extrasynaptic regions.

DISCUSSION

The present results confirm and extend the previous observation that the glutamate sensitivity is localized to the synaptic region of the crayfish muscle (Takeuchi & Takeuchi, 1964). This sensitivity was confined to the subsynaptic membrane since it decreased to less than 5 % 2 μm away from the nerve terminal. A small progressively decreasing sensitivity extended to about 30 μm from the synapse. A similar highly localized synaptic chemosensitivity for ACh has already been found at vertebrate neuromuscular junctions (Kuffler & Yoshikami, 1975). Glutamate sensitivity apparently reflects the high subsynaptic density of glutamate receptors and the more diffusely distributed receptors in the extrasynaptic region. The density of the glutamate receptor may be very low beyond 40 μm from the synaptic terminal and it was difficult to detect the sensitivity with the present experimental technique.

Different actions of kainic acid on the glutamate potentials produced at synaptic and extrasynaptic regions suggest that the pharmacological properties of the synaptic receptors are different from those of extrasynaptic receptors. L-α-kainic acid has a similar but more rigid conformation than that of L-glutamate and it competitively depressed the glutamate action at the synaptic receptors. Since the e.p.s.p. was also depressed by kainate, the above observation supports the view that the neural receptor is identical with the glutamate receptor of the subsynaptic membrane (Takeuchi & Takeuchi, 1964). It is assumed that the synaptic receptor can bind both L-glutamate and L-α-kainate. However, the ionic channel in the subsynaptic membrane is open, only when the synaptic receptor is bound by L-glutamate. Different types of glutamate receptors have been already found in the insect muscles (Cull-Candy, 1976; Gration, Clark & Usherwood, 1979).

The mode of action of kainate on the extrasynaptic receptor was different from that on the synaptic receptor. Kainate enhanced the glutamate action at the extrasynaptic region. While these studies are not complete, it is tentatively assumed that the extrasynaptic receptor has more than one binding site and the ionic channel is open only when these sites are bound by either L-glutamate or L-α-kainate. It seems, however, that at least one binding site should be occupied by L-glutamate. Another possibility would be that several receptors act together to open an ionic channel. In order to test these possibilities it seems essential to examine the dose-conductance curve and to measure its cooperativity. Particularly it is necessary to decide whether or not the cooperativity changes, when kainate is applied. An approximately parallel shift of the dose-response curve along the linear dose axis corresponds to an increase in the agonist concentration, suggesting that the concentration of kainate is additive to glutamate concentration. An increase in the glutamate potential by kainate could also be explained by inhibition of glutamate uptake systems. Since kainic acid acts on other synapses where glutamate causes the depolarization (e.g. de Santis,

Eusebi & Miledi, 1978), kainate may be an interesting drug for the analysis of glutamate action.

If there are two types of receptors in close proximity and an agonist was applied to the bath, or in the case of ionophoretic application, if the applied agonist covered the area of more than several tens μm in diameter, the resulting response would be the sum of the responses due to different receptors (Shinozaki & Shibuya, 1974; Takeuchi & Onodera, 1975; see also Albuquerque & Gage, 1978 for ACh receptor). Although the density of the extrasynaptic receptor is low, the area covered by the extrasynaptic receptor is much larger than that of the subsynaptic membrane, and the resulting response due to the extrasynaptic receptor could be significant.

The glutamate depolarization started within 300 μsec after the glutamate application. Since there are several factors which introduce artificial time lags in the onset of the potential, it is reasonable to consider that real reaction delay must be shorter than 300 μsec. Thus the binding of glutamate to the receptor and opening of the channel may be a fast process comparable to that observed in the ACh receptor (Katz & Miledi, 1965*a*). This observation excludes the possibility that the glutamate potential is the secondary effect due to the release of the endogenous transmitter from the presynaptic nerve terminal (Colton & Freeman, 1975). It has been observed recently that the presynaptic nerve is depolarized by glutamate (Thieffry & Bruner, 1978). Therefore, the glutamate receptor is likely to be present both in the post-synaptic membrane and in the excitatory presynaptic nerve terminals of the crayfish. Since ACh receptor is also found in the presynaptic nerve terminals of many cholinergic systems (for review see Miyamoto, 1978), the presence of receptors on both post-synaptic and presynaptic membranes may be a general phenomenon in the chemical synapses.

The contraction of muscle fibre produced by L-glutamate and by the spontaneous e.p.s.p. suggests that the synaptic membrane is permeable to Ca as observed in the vertebrate neuromuscular junction (Takeuchi, 1963; Kuffler & Yoshikami, 1975; Onodera & Takeuchi, 1976; see Ashley & Campbell, 1978 for barnacle muscle). The slow muscle fibre of the crayfish does not produce the full-sized action potential and the threshold depolarization for the contraction is about 50 mV (Matsumura, 1972). Therefore, the graded contraction resulting from the Ca entry through the synaptic membrane may have a physiological importance.

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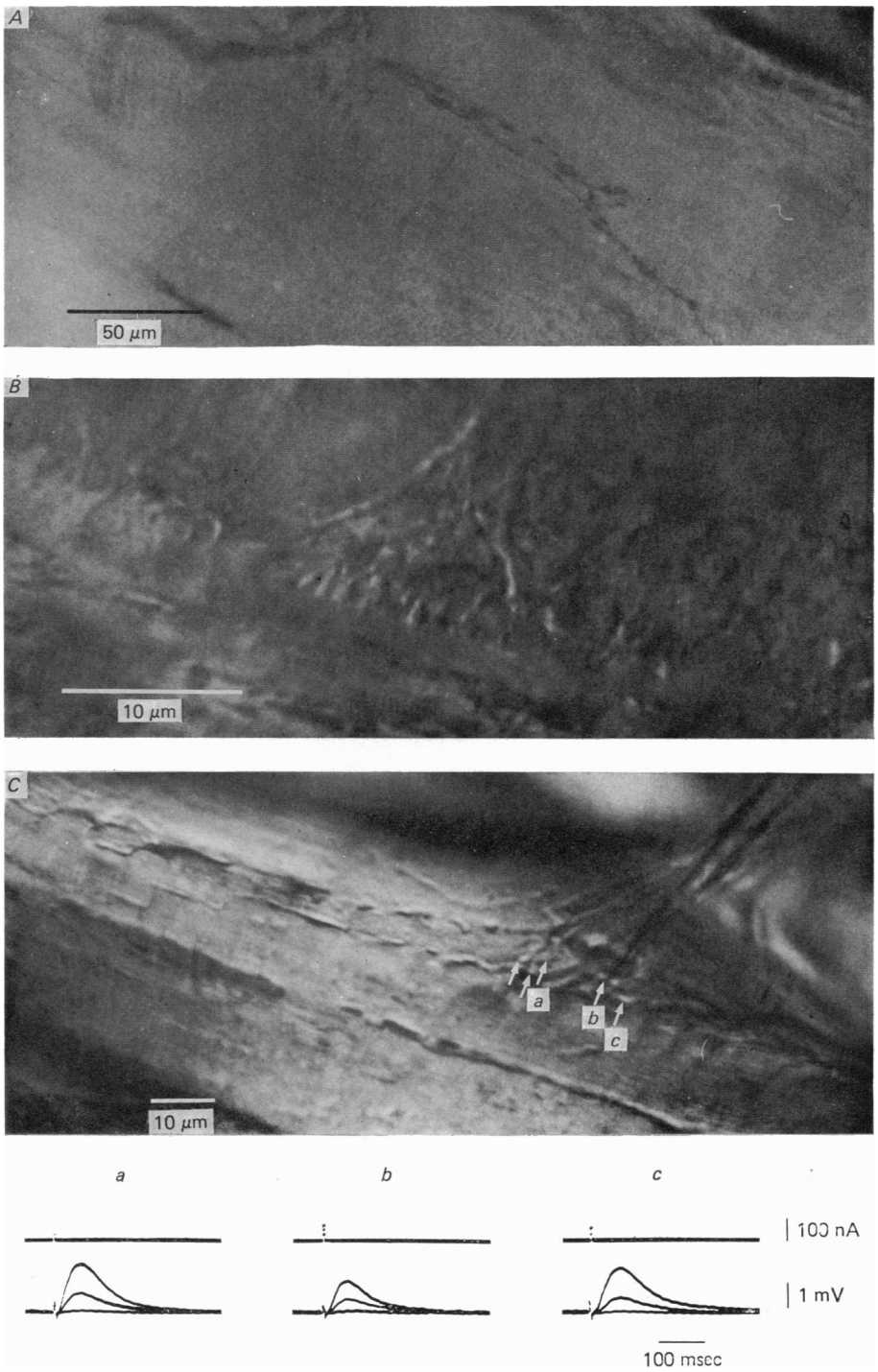
EXPLANATION OF PLATES

PLATE 1

A, opener muscle stained with methylene–blue. Photomicrograph with a 16× objective. *B*, photomicrograph of the synaptic terminal with a 100× oil-immersion objective, recorded from a different muscle fibre. The preparation was treated with collagenase. *C*, photomicrograph with a 40× water immersion objective. Arrows indicate the points where the glutamate response was obtained and sample records are shown in the bottom. A glutamate micro-electrode can be seen on the upper right corner. The muscle was not treated with collagenase.

PLATE 2

A, cluster of boutons in a methylene–blue stained preparation with a 40× water immersion objective. L-Glutamate was applied at points indicated by arrows. Dark arrows indicate the points where the sensitivity was 8–30 mV/nC and open arrows are those where the sensitivity was less than 0·7 mV/nC. Some coloured terminals had already begun to fade. *B*, nerve branches with boutons or varicosities in methylene–blue stained preparation. Dark arrows indicate the points where the sensitivity was 2–4 mV/nC and open arrows, less than 0·2 mV/nC.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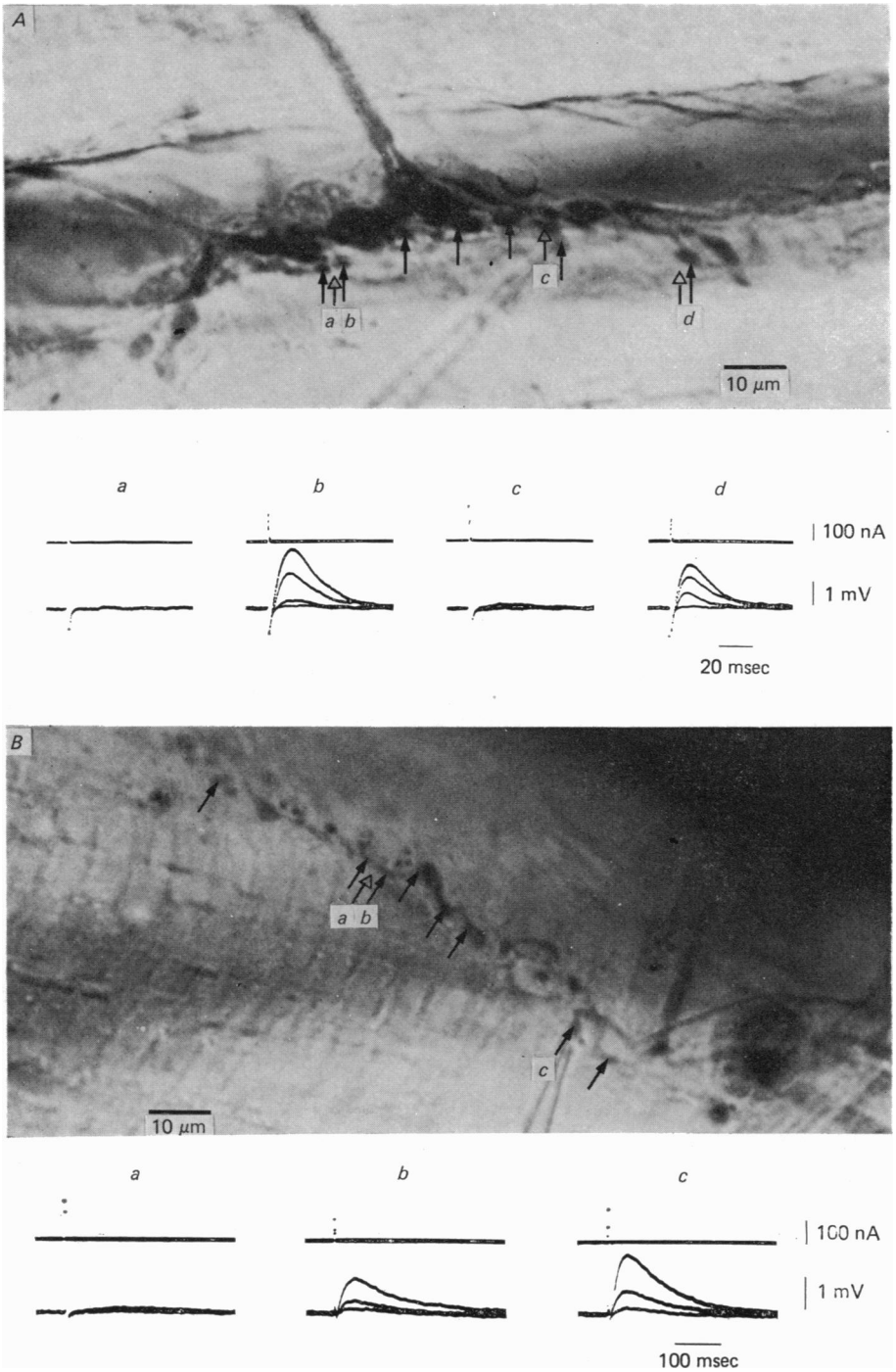
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