J. Physiol. (1985), **369**, *pp.* 183–197
With 7 text-figures
Printed in Great Britain

POST-SYNAPTIC CALCIUM INFLUX AT THE GIANT SYNAPSE OF THE SQUID DURING ACTIVATION BY GLUTAMATE

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(*Received 13 November 1984*)

SUMMARY

1. Changes in free calcium were monitored in the post-synaptic axon of the giant synapse of the squid, using the calcium indicators aequorin and Arsenazo III.

2. The peak size of the calcium-dependent optical signals recorded from aequorin and Arsenazo III both showed a linear relation with the amount of calcium injected ionophoretically into the axon, but the Arsenazo signal had a slower time course than the aequorin.

3. Ionophoretic application of glutamate to the post-synaptic axon depolarized the axon and caused a rise in intracellular free calcium. Aequorin signals were detected in natural sea water, and their size increased when the calcium concentration in the sea water was raised. Arsenazo signals could be detected only in high-calcium (55 mM) sea water.

4. Intracellular calcium signals were detected also during bath application of several glutamate analogues, including kainate, ibotenate, and aspartate.

5. The peak amplitude of the intracellular calcium signal, monitored with both indicators, increased with increasing ionophoretic glutamate dose, and varied linearly with the integral of the glutamate-induced membrane depolarization.

6. No calcium signals were detected when depolarizations, similar to those produced by glutamate, were induced by current injection in the absence of glutamate. We conclude that glutamate increases the calcium permeability of the post-synaptic membrane, independently of the glutamate-induced depolarization.

7. The glutamate-induced depolarization and the rise in intracellular free calcium increased roughly linearly as the membrane potential was made more negative. Extrapolation of these data indicated that the glutamate depolarization would reduce to zero at about −30 mV, while the calcium signals would be suppressed at about +50 mV.

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INTRODUCTION

Using calcium indicators, a calcium influx has been observed across the post-synaptic membrane at the squid giant synapse (Kusano, Miledi & Stinnakre, 1975*b*; Miledi & Stinnakre, 1977) and at the frog end-plate (Miledi, Parker & Schalow, 1980; F. Eusebi, R. Miledi, J. Stinnakre & T. Takahashi, unpublished data), in response to the transmitter released from the presynaptic terminal, or to the application of agonist drugs. This paper presents a more detailed study of the calcium influx into the post-synaptic axon at the squid giant synapse, in response to ionophoretic application of glutamate. Because of the uncertainties still present in the various techniques used for monitoring intracellular free calcium, we made experiments employing two different calcium indicators; the photoprotein aequorin, and the metallochromic dye Arsenazo III (for reviews see; Stinnakre, 1981; Blinks, Wier, Hess & Prendergast, 1982; Thomas, 1982).

METHODS

Preparation and electrical recording

Experiments were carried out during the summers of 1975–1977 at the Zoological Station in Naples, using squid (*Loligo vulgaris*) with mantle lengths of 9–16 cm. The stellar ganglion was isolated with the preganglionic and main post-ganglionic nerves attached as previously described (Miledi, 1967). Measurements were made on the 'distal' synapse to the last giant post-axon (Young, 1939; Miledi, 1967).

Recordings were made in natural or artificial sea water. In some experiments the calcium concentration was increased from 11 to 55 mM by adding the appropriate amount of isotonic (0·39 M) $CaCl_2$ solution. The preparation was continuously perfused with oxygenated sea water, at a temperature of 12–18 °C. The post-synaptic axon was penetrated with a micropipette for injection of calcium indicators (see later), and this served also as the voltage-recording electrode. A KCl-filled micro-electrode was used for current injection. A third pipette filled with 1 M-monosodium glutamate was used to apply glutamate ionophoretically to the post-synaptic membrane. In some experiments the post-synaptic axon was penetrated also by an additional pipette filled with 0·39 M-$CaCl_2$.

Monitoring of intracellular calcium with aequorin

General techniques were as previously described (Kusano *et al.* 1975*a*, *b*; Stinnakre, 1981). Briefly, about 1 mg of aequorin (a gift from O. Shimomura) was desalted on a small Sephadex G-25 fine column and eluted with a buffer containing 250 mM-KCl, 5 mM-HEPES and ethylenediaminetetraacetic acid (EDTA; 5×10^{-6} to 5×10^{-5} M). The pH was adjusted to 7·8 with KOH. The few drops eluted from the column were collected separately in quartz or plastic tubes and stored at *ca.* 4 °C. Attempts to use less EDTA gave poor results, probably because aequorin was destroyed during preparation or injection into the giant axon.

Aequorin was injected under pressure in the dark from either single- or double-barrel micropipettes made by hand on a microforge, using thoroughly washed borosilicate glass tubing. When using a single-barrel pipette, electrical recording of the membrane potential was made using an Ag–AgCl wire making contact with the aequorin solution in the pipette tip via a 'bridge' of buffer solution. In the double-barrel pipettes, the pressure injection barrel was filled with aequorin, whilst the other contained 3 M-KCl and was used for electrical recording. The aequorin pipette was left in position throughout an experiment, but was painted with opaque laquer to shield the light emitted from the pipette. Light emitted from the preparation was measured by a photomultiplier (Hamamatsu R374), located with the photo-cathode just above the fluid surface. The anode current was measured via a current to voltage converter, and filtered by a simple RC circuit (time constant 5–600 ms).

Monitoring of intracellular calcium with Arsenazo III

Techniques for dye injection and optical recording of calcium-dependent changes in Arsenazo absorbance were as described previously (Miledi & Parker, 1981). Calcium-dependent changes in light absorption of the dye were monitored at a wave-length of 650 nm, and are expressed in terms of fractional changes in transmitted light (ΔI). Ionophoretic currents of several hundred nA were required to inject the dye over a time of some tens of minutes. Loading of dye was followed by eye through a dissecting microscope, and was stopped when the synaptic region of the axon was clearly stained. Dye pipettes were found to block readily in the high-calcium sea water, so the pipettes were inserted whilst bathing in normal sea water, and perfusion with high-calcium sea water was started once the axon was loaded.

RESULTS

Injection of indicators

In early experiments, attempts were made to inject Arsenazo III (Sigma Chemical Co., practical grade) which had not been treated to remove contaminating calcium. The injected dye in these cases was seen to be localized in long thin 'sacs' spreading distally from the injection site, similar to those seen previously after injection of some Procion dyes (R. Miledi, unpublished). The Arsenazo III in the sacs was blue, suggesting a high level of free calcium, but over a period of hours spread into the bulk of the axon, where a red colouration, indicative of a low free-calcium level, became visible. This phenomenon was not seen using Arsenazo treated with chelex 100 (Bio Rad), to remove contaminating calcium (Miledi & Parker, 1981), where the dye appeared to diffuse uniformly through the axon, and was a red colour. All results described here were obtained with Arsenazo III treated in this way.

The presence of aequorin in the axon could not be seen directly, but it appeared to spread quite quickly throughout the synaptic region, since light emission to either intracellular calcium pulses or extracellular glutamate application was detected within less than 30 min after starting injection. 'Good' preparations had a low resting light emission, which did not increase appreciably when the calcium concentration in the bathing solution was raised from 11 to 55 mM, or when the membrane potential was stepped to more negative voltages (*ca.* -140 mV).

Responses of aequorin and Arsenazo III to intracellular calcium injections

Ionophoretic application of glutamate from a localized pipette to the post-synaptic axon is expected to activate only a small patch of membrane. Thus, the calcium influx across this localized area should approximate a point source, and experiments described later using Arsenazo III indicated that this was the case. To study the characteristics of the two calcium indicators under similar conditions of a localized increase in free calcium, we used intracellular injections of calcium from a micropipette filled with $CaCl_2$ solution. Injection was made by ionophoresis, but because of the low input resistance of the post-synaptic axon, even large injection currents caused only small (< 10 mV) depolarizations. Similar, or larger, depolarizations elicited by passing currents through KCl-filled pipettes did not give detectable responses with either indicator.

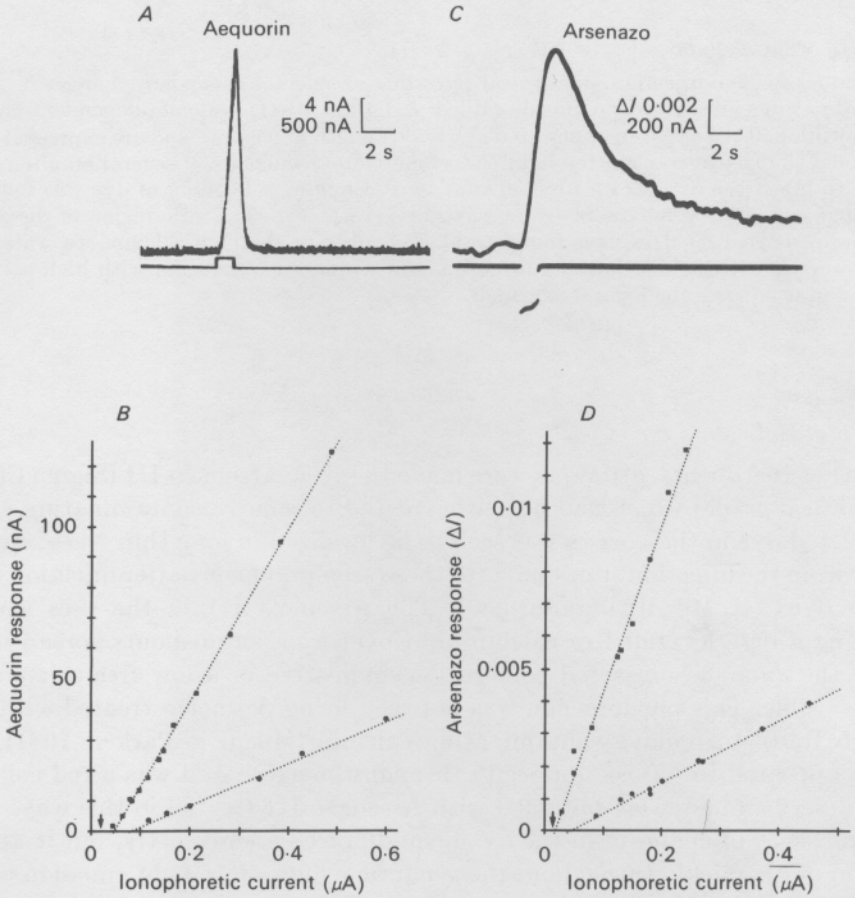

Fig. 1. Aequorin and Arsenazo III responses to injection of calcium pulses into post-synaptic axons. Examples of recordings obtained with aequorin (*A*) and Arsenazo III (*C*) using similar calcium pulses. The upper trace in each frame shows the optical signal from the calcium indicator. The aequorin trace indicates calcium-dependent light emission, measured as the photomultiplier anode current. The Arsenazo trace shows calcium-dependent changes in dye absorption at 650 nm, and the calibration gives the fractional change in transmitted light intensity (ΔI). In this, and other Figures, upward deflexions in both aequorin and Arsenazo traces correspond to increases in free-calcium concentration. The lower traces show the ionophoretic current monitor. Pipette positive currents correspond to upward deflexions in aequorin experiments and downward deflexions in Arsenazo experiments. *B*, *D*, peak amplitude of aequorin (*B*) and Arsenazo III (*D*) signals plotted against ionophoretic calcium current. Pulse durations were; 0·18 and 0·85 s (aequorin) and 0·2 and 1 s (Arsenazo). Arrows indicate the backing currents applied to the calcium pipettes.

With aequorin, the calcium pulse gave a transient increase in light emission (Fig. 1*A*), the peak amplitude of which increased linearly with the charge carried during the ionophoretic current (Fig. 1*B*) for durations up to at least 1 s (see also Kusano *et al.* 1975*a*, *b*). After an initial lag lasting several milliseconds, the aequorin light rose linearly during the pulse, and then fell rapidly to the base line soon after the end of the pulse. The decay phase was approximately exponential, with a half decay time of between 0·04 and 0·14 s.

Pulses of calcium injected into post-synaptic axons loaded with Arsenazo III gave slow transient increases in light absorbance at 650 nm (Fig. 1*C*), while at 530 nm small decreases were seen, as expected if these changes resulted from calcium-dependent changes in dye absorption (Miledi & Parker, 1981; Miledi, Parker & Zhu,

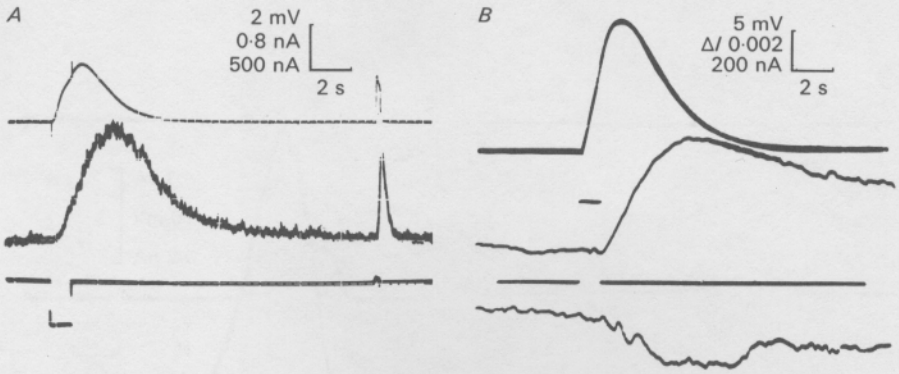

Fig. 2. Aequorin (*A*) and Arsenazo III (*B*) responses to ionophoretic application of glutamate to the post-synaptic membrane. *A*, traces show, from top to bottom; post-synaptic potential, aequorin light emission and ionophoretic current monitor. Experiment in normal sea water, at 13 °C. The first response was elicited by glutamate application, and the second by ionophoretic injection of calcium into the axon. *B*, traces show, from top to bottom; post-synaptic potential, Arsenazo light absorbance at 650 nm, ionophoretic current monitor and light absorption at 532 nm. High-calcium sea water, at 16 °C.

1982). The time course of the Arsenazo response was slower than with aequorin, reaching a peak about 1 s after the end of the pulse, and then decaying over a period of several seconds. Half decay times of between 1 and 4 s were observed in three experiments, with the decay time increasing for larger calcium injections. These values are shorter than those observed for Arsenazo responses to calcium injection into the presynaptic axon under similar conditions (6–13 s; Miledi & Parker, 1981).

When the measuring light spot was moved away from the calcium pipette, so that the edge of the spot (diameter *ca.* 200 μm) was just clear of the tip of the pipette, the Arsenazo response became barely detectable. With the pulses of calcium used, diffusion of calcium ions into the axoplasm must therefore have been restricted to a small region around the pipette tip of no more than about 50 μm. A similar spatial restriction of calcium action was observed with the release of transmitter evoked by ionophoretic injection of calcium from one or two pipettes inserted into the presynaptic nerve terminal (Miledi, 1973; and unpublished observations).

As with aequorin, the relationship between the peak Arsenazo signal size (at 650 nm) and the total charge passed through the ionophoretic calcium pipette was linear, for pulse durations up to at least 1 s (Fig. 1*D*).

Calcium signals to ionophoretic glutamate application

Ionophoretic application of glutamate pulses in the region of the synaptic contact produced transient depolarizations of the post-synaptic membrane (Miledi, 1967, 1969), which were accompanied by aequorin and Arsenazo III signals (Fig. 2). The

depolarizing responses must have arisen from an action of glutamate on the post-synaptic membrane, and not indirectly as a result of transmitter release from the presynaptic terminal, because similar responses to glutamate can be recorded from ganglia where the presynaptic terminal has degenerated following section of the presynaptic nerve (A. de Santis, A. Miralto & R. Miledi, unpublished observa-

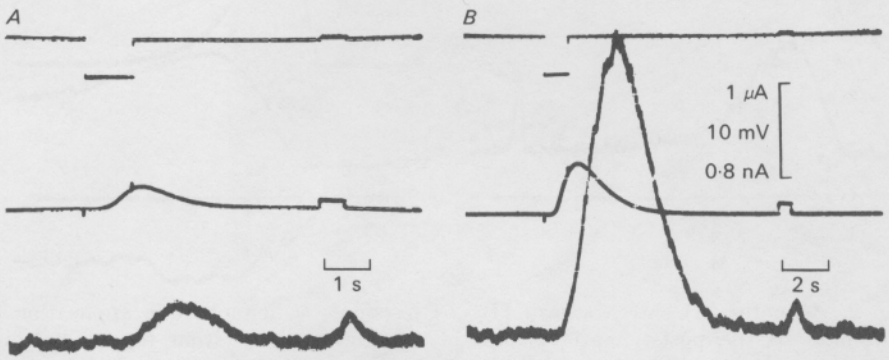

Fig. 3. Glutamate-induced depolarizations and aequorin signals recorded from the same axon bathed in normal sea water (*A*) and high-calcium (55 mM) sea water (*B*). Top trace is current monitor, middle trace is membrane potential and lower trace aequorin light emission. A fixed pulse of calcium was applied intracellularly near the end of each record, and the constant aequorin response indicates that the aequorin sensitivity did not change in the 20 min interval between the two records. The input resistance of the axon increased by about 35 % after changing to the high-calcium sea water. Membrane potential in both records was about −66 mV. Temperature was 12·5 °C.

tions). Furthermore, the calcium indicator signals did not arise as any consequence of the depolarization *per se*, since responses were not detected to similar depolarizations produced by injecting current into the post-axon through KCl pipettes. This can be seen in Fig. 5, where a glutamate-induced depolarization of 6 mV was accompanied by a clear aequorin response (Fig. 5*B*), although a depolarization of 13 mV produced by current injection through a KCl pipette failed to give any signal (Fig. 5*A*).

Aequorin responses

The over-all time course of the glutamate-induced light emission was superficially similar to that of the glutamate depolarizing response, although with a longer onset and a later time to peak (Fig. 2*A*). After the membrane potential had returned to the base line, the light signal decayed roughly exponentially, with a half-time of about 1 s. Occasionally, one or more humps were visible on the depolarizing response, suggesting that glutamate was acting at two or more spots with a high density of glutamate receptors. These humps were not observed on the light records.

Aequorin responses could be obtained in sea water containing a normal calcium concentration (Fig. 3*A*). When the perfusion fluid was changed from normal sea water to high-calcium sea water, the light response to a constant glutamate pulse increased by a factor of eight (mean = 8·05 ± 1·36; S.E. of mean, nine ganglia), whilst the glutamate depolarization doubled (mean = 2·05 ± 0·13; thirteen ganglia; see also

Fig. 3*B*). Responses to intracellular injections of calcium were not altered by these changes in external calcium (Fig. 3).

By roughly matching a given glutamate light response with an intracellular pulse of calcium, adjusted so as to elicit responses with similar rates of rise, an approximate estimate could be made of the fraction of the total glutamate current which is carried by calcium ions (cf. Kusano *et al.* 1975*b*). In normal sea water a value of 1·4–3 % was obtained. The peak glutamate-induced current was estimated by polarizing the membrane such that the potential at the peak of the glutamate response returned to the original resting potential (e.g. Fig. 5*C*); thus, the imposed polarizing current would be equal to the glutamate-induced current. The transport number of the calcium pipettes was assumed to be 0·1 (Kusano *et al.* 1975*a*).

Arsenazo III

In contrast to aequorin, responses with Arsenazo III were not detectable during even large applications of glutamate to axons bathed in normal sea water. However, responses were readily obtained from the same axons after changing to high-calcium sea water. This is similar to the situation at the frog end-plate, where in normal-calcium (1·8 mM) Ringer it was difficult to detect Arsenazo signals (Miledi *et al.* 1980) even though aequorin signals were readily obtained (F. Eusebi, R. Miledi, J. Stinnakre & T. Takahashi, unpublished data). Fig. 2*B* shows the changes in light absorption at 650 and 532 nm which accompanied glutamate activation of an Arsenazo III loaded synapse bathed in high-calcium (55 mM) sea water. Similar to the calcium injections, the calcium which entered the axon in response to glutamate was confined to a sharply localized area, and the size of the Arsenazo response was greatly reduced if the measuring light spot was moved to be just clear of the tip of the glutamate pipette.

As with aequorin, a latency was apparent between the beginning of the glutamate potential response and the beginning of the Arsenazo response. This can be seen in Fig. 2*B*, where the light response began about 1 s after the onset of the glutamate potential. The latency did not appear to vary with different sizes of glutamate pulses, and values of between 0·5 and 1 s were observed in different experiments. These delays cannot be attributed to the response times of either Arsenazo III or aequorin, both of which are faster by a factor of at least one hundred times (see Blinks *et al.* 1982).

The decay of the Arsenazo response varied with the size of the glutamate pulse, and increased with larger doses. Half decay times of between 3·5 and 9 s were recorded for the range of pulses used, which elicited depolarizations up to 10 mV. Occasionally, it was noted that when a large number of glutamate pulses had been applied to one area, the decline of the Arsenazo response became markedly slower. This slowing of the decay was localized to a small region of the synapse, and responses with normal decay times could be obtained after re-positioning the tip of the glutamate pipette and the measuring light spot a few hundred micrometres away. Presumably, the slowing of the decay arose because the calcium influx was sufficient to locally saturate the calcium-buffering and uptake systems of the axon. Aequorin recordings did not show this slowing, but this may have been because the greater sensitivity of this indicator allowed us to use smaller glutamate doses.

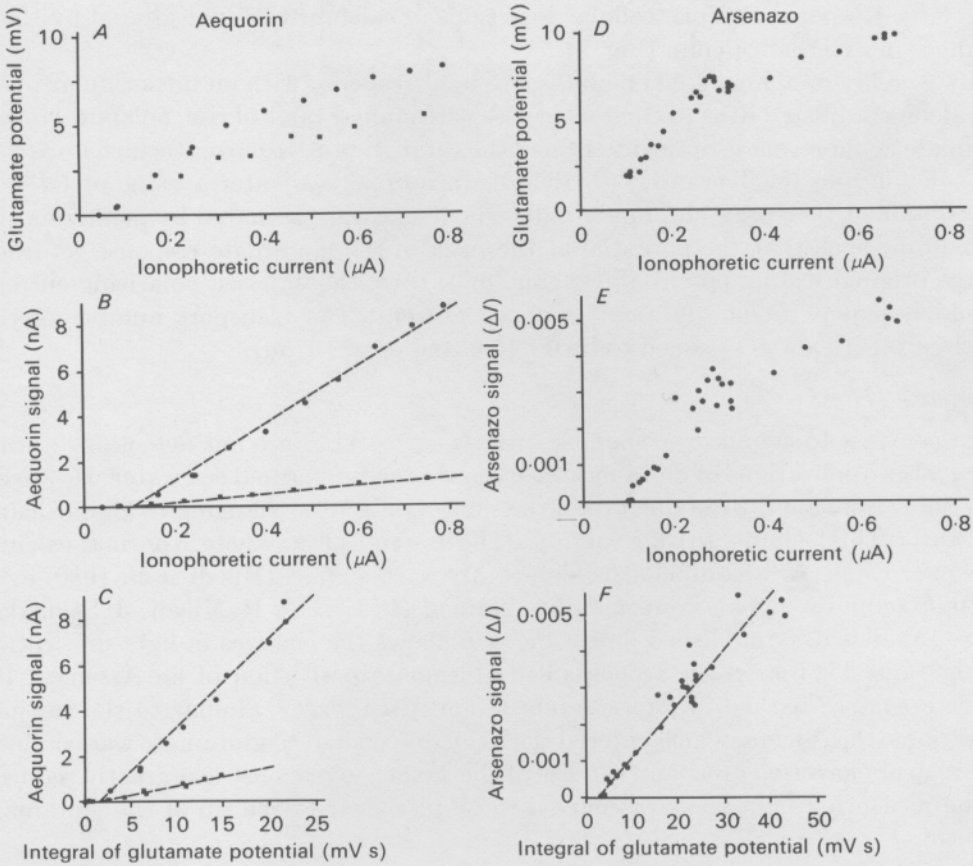

Fig. 4. Dose–response relationships for the glutamate-induced calcium signals. Two experiments with aequorin (*A–C*), and one experiment with Arsenazo III (*D–F*) are shown. In all cases, ionophoretic glutamate pulses of 1 s duration were used. Aequorin recordings were obtained in normal sea water and Arsenazo III recordings were obtained in high-calcium (55 mM) sea water. *A*, *D*, peak amplitudes of depolarizations (ordinate) elicited by different ionophoretic glutamate currents. *B*, *E*, peak amplitudes of aequorin (*B*) and Arsenazo (*E*) signals against glutamate current. *C*, *F*, peak amplitudes of the aequorin (*C*) and Arsenazo (*F*) signals plotted against the time integral of the glutamate-induced depolarization up to the time of the peak calcium response. Regression lines are fitted to the data.

Glutamate analogues

Bath application of L-glutamate elicited a small depolarization of the post-synaptic axon, which was accompanied by a slow rise in aequorin light emission (cf. Kusano *et al.* 1975*b*; Miledi & Stinnakre, 1977). Other substances, chemically related to glutamate, gave similar responses. Kainate, ibotenate, D-glutamate and D- and L-aspartate all gave depolarizations and aequorin–calcium signals when applied in high-calcium (55 mM) sea water. Kainate had roughly the same potency as L-glutamate, while the other substances were less effective.

Dose–response characteristics

In all experiments, the depolarization induced by glutamate varied as a graded function of the ionophoretic current, although the response tended to level off with large pulses (Fig. 4*A*, *D*). This behaviour was similar in normal and high-calcium sea water.

The size of the Arsenazo signal varied with glutamate dose in the same way as the depolarizing response (Fig. 4*E*), while the aequorin signal was more nearly a linear function of the glutamate dose. Using Arsenazo III, a glutamate dose sufficient to give an appreciable depolarization was required before a detectable light signal was obtained (Fig. 4*E*). A similar threshold was seen with aequorin responses in normal sea water, but in high-calcium sea water the aequorin light response became detectable at glutamate doses giving a just detectable depolarization.

Since the peak amplitude of the response with both aequorin and Arsenazo appears to reflect the total entry of calcium for durations up to at least 1 s (see earlier), we examined the relation between the indicator responses and the integral under the potential response up to the time of the peak light response. The electrical time constant of the post-synaptic axon is very fast ($<$ a few milliseconds) compared to the time course of the glutamate-induced depolarizations, so that the potential will reflect the underlying membrane current, and its integral will give a measure of the total charge movement across the membrane. Plots of the indicator responses against integral of the potential are shown in Fig. 4*C* for aequorin and Fig. 4*F* for Arsenazo III. Both of these show a linear relationship. However, regression lines fitted to the data intercept the potential area axis, further suggesting that a finite calcium influx was required to give a detectable signal.

Voltage dependence of glutamate potential and calcium responses

Changes in membrane potential would be expected to alter the size of the glutamate-induced depolarization by changing the driving force for the movement of sodium and potassium ions, which carry the major part of the synaptic current (Miledi, 1969), and to alter the aequorin and Arsenazo responses by changing the driving force for calcium influx. Kusano *et al.* (1975*b*) observed that the synaptically evoked depolarization showed a much steeper dependence upon membrane potential than did the accompanying aequorin responses. We repeated these measurements, using ionophoretically applied glutamate and monitoring calcium influx with aequorin and Arsenazo.

Fig. 5 shows records of glutamate-induced responses obtained when the membrane potential was displaced to different steady levels by injecting currents through an intracellular KCl-filled pipette. Measurements from experiments such as these are shown in Fig. 6, using aequorin (*A*, *B*) and Arsenazo III (*C*, *D*) as the calcium indicators. The voltage dependence of the glutamate-induced depolarization was not linear, but usually showed a marked convex curvature at high negative potentials (Fig. 6*A*, *C*). A rough extrapolation from the observed data points, using curves drawn by eye, indicated a reversal potential for the glutamate potential of about -30 mV. Miledi (1969) directly measured a similar reversal potential for glutamate.

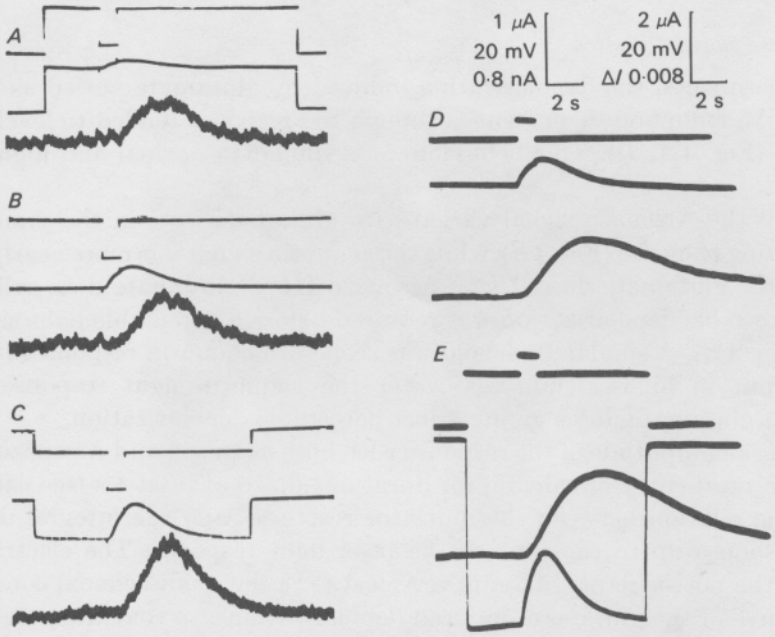

Fig. 5. Effect of changes in membrane potential on the responses elicited by ionophoretic application of glutamate. In each frame, records show (from top to bottom); current monitor, membrane potential and calcium indicator signal. The current monitor includes currents through both the ionophoretic glutamate pipette, and the intracellular current injection pipette used to displace the membrane potential. *A–C*, records at three different holding potentials, from an experiment using aequorin. Resting potential at the beginning of each trace was −55 mV. High-calcium sea water at 12·5 °C. *D*, *E*, records at two potentials from an experiment with Arsenazo III. Resting potential was −61 mV. High-calcium sea water at 15 °C.

No clear change in the extrapolated reversal potential was apparent when changing from normal- to high-calcium sea water.

In contrast to the glutamate-induced depolarization, both the aequorin (Fig. 6*B*) and Arsenazo (Fig. 6*D*) signals varied approximately linearly with membrane potential, decreasing in size as the potential was made less negative. The voltage dependence of the peak calcium signals was less steep than that of the depolarization. Extrapolation of regression lines, to the potentials which would give zero calcium signals, gave mean values of +48 mV for aequorin (four ganglia), and +46 mV for Arsenazo III (four ganglia).

Strong hyperpolarization, to potentials more negative than *ca.* −150 mV often elicited calcium signals from both aequorin and Arsenazo III. This probably arose because of electrical breakdown of the membrane, leading to influx of calcium ions.

Desensitization of the glutamate responses

It is known that the glutamate receptors in the membrane of the squid axon desensitize easily during repeated or maintained glutamate application (Miledi, 1967). To ensure good repeatability, records were therefore usually obtained using

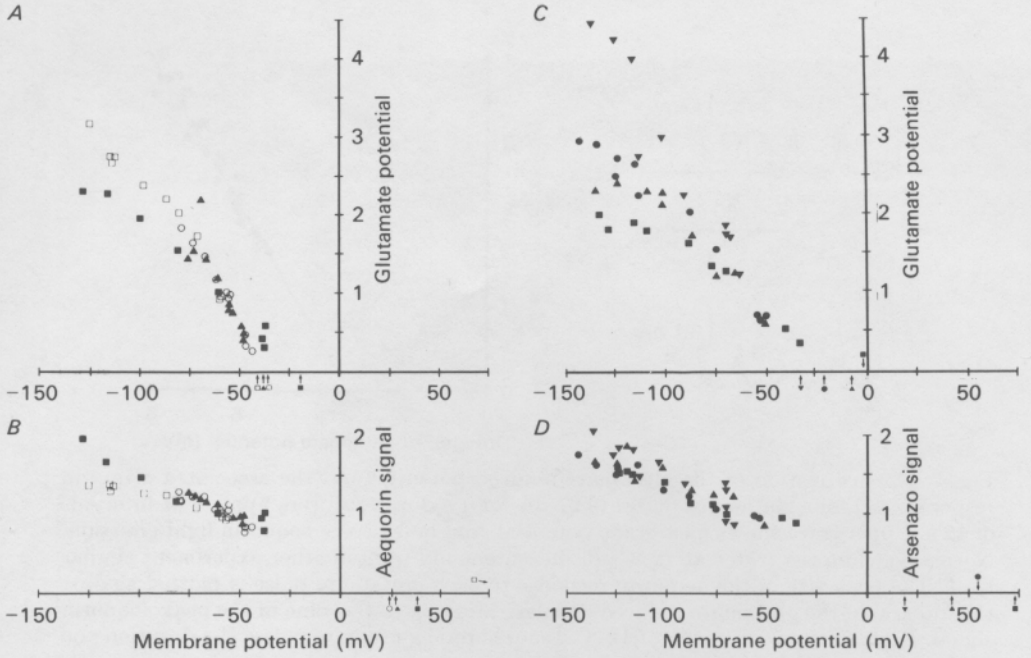

Fig. 6. Voltage sensitivity of the glutamate-induced potential (*A*, *C*), and of the associated aequorin (*B*) and Arsenazo (*D*) responses. In each experiment, the glutamate potentials are plotted as a fraction of the values obtained at the resting potential. The calcium indicator signals are similarly scaled. Several experiments are shown, using different glutamate pulse intensities and durations. Open symbols indicate experiments in normal sea water, filled symbols show experiments in high-calcium sea water (squares are from the same preparation). The extrapolated X-axis intercepts of regression lines fitted to the data from each experiment are indicated by the small symbols.

an interval of a few minutes between each test. When glutamate pulses were applied at intervals of 30 s or shorter, both the glutamate-induced potentials and aequorin light signals progressively declined (Fig. 7*A*). This phenomenon was apparent also with Arsenazo III, but in this case interpretation was complicated because the slow decay of the signal caused summation of successive responses. The relationship between the peak size of the aequorin signal and the integral of the glutamate depolarization during repetitive pulses (Fig. 7*B*) was similar to the relation seen with different sizes of glutamate pulse (Fig. 4*C*).

DISCUSSION

Calcium measurements using aequorin and Arsenazo III

The calcium indicators aequorin and Arsenazo III both showed a linear relationship between the total amount of calcium injected into the axon from an ionophoretic pipette and the peak size of the indicator light responses. In the case of Arsenazo III, these results confirm similar measurements made on frog muscle fibres (Miledi *et al.* 1980), *Aplysia* neurones (Gorman & Thomas, 1978) and squid presynaptic terminals (Miledi & Parker, 1981). A linear relationship is also expected from *in vitro*

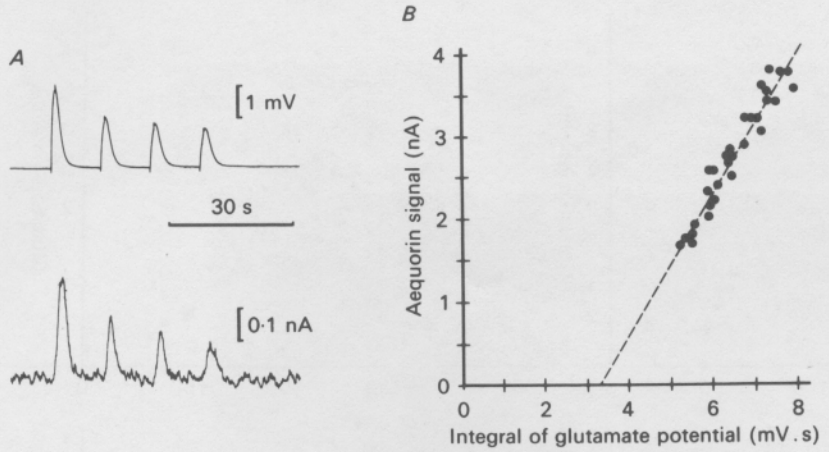

Fig. 7. Desensitization of the glutamate-induced potential and the associated aequorin responses. *A*, four glutamate pulses (0·27 μA for 1 s duration) were applied at intervals of 12 s. Upper trace shows membrane potential, and lower trace aequorin light emission. Normal-calcium sea water at 12 °C. *B*, measurements from another experiment similar to *A*. The peak size of the aequorin response to each glutamate pulse is plotted against the integral of the glutamate-induced depolarization up to the time of the peak aequorin signal. High-calcium sea water, at 12 °C. Ionophoretic glutamate pulses (1 s duration and 300 nA) were applied at 30 s intervals.

calibrations of the dye (see Thomas, 1982; Blinks *et al.* 1982). The results obtained with aequorin, although in agreement with observations of calcium injections into droplets of aequorin (Kusano *et al.* 1975*a*) and frog muscle fibres (F. Eusebi, R. Miledi & T. Takahashi, unpublished observations), differ from results obtained by rapid mixing of aequorin with calcium-buffered solutions (see Blinks *et al.* 1982), where a power law relationship is observed.

The reason for this difference is not clear, but we can consider three possibilities. (i) If the increment in free calcium concentration resulting from the injection was much lower than the resting free calcium level, then a linear relation of the aequorin response with the amount of injected calcium would be expected (Baker, Hodgkin & Ridgway, 1971). This was not the case in our experiments, or those of Kusano *et al.* (1975*a*), since the resting aequorin glow (which arose from a large volume of the axon) was generally smaller than the aequorin signal produced by the localized calcium ejected from the pipette tip. (ii) The calcium levels resulting from the ionophoretic injections might have been sufficiently low so as to lie on the 'foot' of the aequorin–calcium dose–response curve, where an approximately linear relation holds. This seems unlikely for two reasons: First, a linear relation was observed over a more than tenfold range of calcium doses (Fig. 1*B*), while the dose–response curve approximates to linear only over a smaller range (Fig. 4 of Blinks *et al.* 1982). Secondly, quite large aequorin responses were elicited, which must have arisen from a restricted volume of aequorin around the pipette tip. The calcium concentration in this region was almost certainly much above the linear part of the dose–response curve (*ca.* 10^{-7} M). (iii) The calcium level in the localized region around the pipette tip may have been high enough to 'burn' all the immediately available aequorin, so that the linear relation arose because of the extremely steep spatial distribution. We feel that this is the most likely explanation.

Thus, for any situation where calcium entry or release into a cell is expected to occur from a localized source, a linear calibration of the peak aequorin response in terms of free calcium would seem more appropriate than a power law. Certainly, this

seems to be the case in the present experiments, where calcium entry was induced by focal application of glutamate. Evidence for this is that the peak aequorin light response was linearly related to the area under the glutamate response (Fig. 4*C*), as was the Arsenazo III response, the linearity of which is well established.

In the present experiments the sensitivity of Arsenazo III appeared to be less than that of aequorin. For example, we were unable to detect Arsenazo responses to glutamate without raising the calcium concentration in the sea water, while aequorin responses were readily detectable in normal sea water. Also, the Arsenazo responses to glutamate application were only seen once the activation exceeded some threshold value (Fig. 4*F*), while this phenomenon was less marked with aequorin. We do not understand this threshold effect, but one possibility arises from the fact that the post-synaptic receptors are located on membrane processes, forming 'sacs' linked to the main axon by narrow necks (Martin & Miledi, 1978). Glutamate-induced calcium influx might therefore build up highly localized regions of elevated free calcium, which would favour the aequorin response over that for Arsenazo III (see Blinks *et al.* 1982 for discussion).

A conspicuous difference between the two calcium indicators was that, for both ionophoretic calcium pulses and the glutamate-induced calcium entry, the time courses of decline of the light signals were about five times slower with Arsenazo than for aequorin. This cannot be due to any differences in response speeds of the indicators, since both are very much faster than the fastest decays observed. Possible explanations may include; (i) the decay phase of the aequorin response could reflect the 'power law' relationship between light emission and free calcium level, (ii) sufficient Arsenazo III may have been injected to act as an appreciable calcium-buffering system, thus slowing down the calcium transient, and (iii) Arsenazo III may have unspecific effects on the various systems which buffer and actively transport calcium in the axon.

Calcium entry during glutamate activation

Synaptic transmission at the giant synapse is accompanied by an increase in free calcium in the post-synaptic terminal (Katz & Miledi, 1967; Kusano *et al.* 1975*b*). The identity of the transmitter is still not clear, but it is clear however, that application of glutamate also leads to an increase in intracellular free calcium. This increase in calcium does not depend on the glutamate-induced depolarization *per se*, since equivalent or larger depolarizations in the absence of glutamate did not induce the rise in intracellular calcium. The voltage-activated calcium channels described in the squid axon by Baker *et al.* (1971) are, therefore, not involved in the glutamate-activated calcium response. Furthermore, the aequorin and Arsenazo responses elicited by application of glutamate appear to result from a calcium influx, rather than a release from internal stores, since both responses are dependent upon the external calcium concentration, and the calcium signals are proportional to the glutamate-induced current. Thus, the rise in intracellular calcium almost certainly occurs because glutamate directly activates membrane channels which are permeable to calcium ions. Ashley & Campbell (1978) have previously shown that glutamate causes a rise in intracellular calcium in barnacle muscle fibres, but they did not determine whether this was secondary to the membrane depolarization.

The contribution of calcium ions to the total glutamate-activated current appears to be small (roughly 2% in normal sea water), similar to the frog end-plate, where calcium ions carry a small fraction of the post-synaptic current (Miledi *et al.* 1980), even though a different neurotransmitter is involved. Although, in both cases, calcium ions carry a negligible fraction of the post-synaptic current, it is possible that the transmitter- evoked rise in intracellular calcium may play a regulatory role in the establishment and maintenance of synaptic contacts. The finding that kainic acid also causes a rise in intracellular calcium in the post-synaptic axon may provide an explanation for the neurotoxic actions of this glutamate analogue (Olney, Rhee & Ho, 1977).

Calcium entry across the post-synaptic channels increased with hyperpolarization. Over the potential range which we explored (−30 to −150 mV), this voltage dependence was linear using both indicators, and was less steep than the voltage dependence of the glutamate-induced depolarization. This behaviour contrasts with that at the frog end-plate, where the calcium entry activated by acetylcholine increased exponentially with hyperpolarization (Miledi *et al.* 1980 using Arsenazo; F. Eusebi, R. Miledi, J. Stinnakre & T. Takahashi, unpublished observations, using aequorin).

Extrapolation of the aequorin and Arsenazo data suggested that the calcium entry would become zero at a membrane potential of about +50 mV. Obviously, there are several possible errors in making this estimate; (i) the voltage dependence may not remain linear beyond the voltages actually observed; (ii) the 'threshold' effect with Arsenazo III would tend to underestimate the intercept; and (iii) the measurements were not made under voltage clamp, and therefore there were uncertainties in determining the membrane potential prevailing during a response (although this error would be fairly small, since small glutamate responses were used, and the voltage dependence is shallow).

The extrapolated potential for suppression of calcium entry gave a value less positive than would be expected for the calcium equilibrium potential. For example, calcium suppression potentials between +80 and +200 mV have been recorded for transmitter release in the presynaptic terminal of the giant synapse (Katz & Miledi, 1967; R. Miledi, unpublished data), and measurements using calcium indicators (Requena, DiPolo, Brinley & Mullins, 1977) and calcium-selective electrodes (Baker & Umbach, 1983) indicate that the free calcium concentration in the squid giant axon is 10^{-7} M or less. In contrast, values for the suppression potential in the present experiments would suggest an internal free calcium concentration of around 10^{-4} M (but see also Kusano *et al.* 1975*b*). Although we do not know the intracellular free calcium concentration in our experiments, it must have been very much lower than 10^{-4} M, otherwise the resting glow with the aequorin would have been high and the aequorin would have been quickly 'burned'. The possibility remains, however, that the internal calcium level increased greatly in localized spots close to activated channels, so as to reduce the calcium influx. In any case, such a linear extrapolation would only be valid if the glutamate-activated channel displays a constant calcium conductance with voltage, rather than a constant permeability (Hagiwara & Byerly, 1981). The different voltage dependence of calcium entry seen at the squid synapse

and the frog end-plate indicates differences in the way in which calcium ions permeate through these channels, and might, for example, reflect differences in the binding of calcium ions within the channels.

We are grateful to the staff of the Stazione Zoologica of Naples for their constant help and encouragement, to Dr O. Shimomura for a gift of aequorin, and to the Royal Society of London for support.

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