J. Physiol. (1980). **321**, *pp.* 195–210
With 8 text-figures
Printed in Great Britain

SINGLE GLUTAMATE-ACTIVATED CHANNELS RECORDED FROM LOCUST MUSCLE FIBRES WITH PERFUSED PATCH-CLAMP ELECTRODES

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(*Received 10 April 1981*)

SUMMARY

1. Glutamate-activated single channels have been examined with conventional and internally perfused patch-clamp electrodes applied to the extrajunctional membrane of locust muscle fibres which were usually treated with concanavalin A to reduce desensitization. Channels opened by glutamate and other agonists have been compared.

2. Recording patches were selected where there appeared to be only one active channel under the pipette. The conductance for single glutamate-activated channels was 150 pS and was not markedly dependent on clamp potential. The lifetimes of the channels were usually exponentially distributed with a mean of τ(glutamate) = $2{\cdot}3 \pm 0{\cdot}12$ msec, $T = 23$ °C, $V_m = -60$ mV.

3. Channels opened by fluoroglutamate had a mean lifetime of τ(fluoroglutamate) = $1{\cdot}4 \pm 0{\cdot}1$ msec; channels opened by quisqualate had a mean lifetime of τ(quisqualate) = $6{\cdot}4 \pm 1{\cdot}0$ msec. The conductances of channels opened by fluoroglutamate, quisqualate and glutamate were not significantly different.

4. The behaviour of individual receptor–channel complexes has been examined at various concentrations of glutamate. Drug solutions were applied through an internal perfusion pipette which allowed exchange of the solution in the patch-electrode tip within 10 sec. The distribution of channel closed times could be fitted with a single exponential. Channel lifetime was not markedly dependent on glutamate concentration (30–600 μm) whereas the channel closed time decreased with increasing glutamate concentration.

5. The reciprocal of channel closed time *vs.* glutamate concentration had a slope value of 1·85 on logarithmic co-ordinates. The approximately second power dependence of *net* forward reaction rate on glutamate concentration suggests that at least two glutamate molecules activate a single receptor–channel complex.

6. The apparent dissociation constant for the glutamate–receptor complex is large, being about 300–500 μM. If the receptors have an equally low affinity for neurally released transmitter, then only a small amount of the transmitter packet is expected to bind to receptors. Quisqualate and glutamate have similar receptor affinities whereas receptor affinity for fluoroglutamate is smaller.

INTRODUCTION

Techniques such as noise analysis have allowed the estimation of synaptic channel properties at vertebrate (Katz & Miledi, 1972; Anderson & Stevens, 1973) and invertebrate nerve–muscle junctions (Crawford & McBurney, 1976*b*; Anderson, Cull-Candy & Miledi, 1976). More recently the patch-clamp method has allowed a more direct estimate of parameters of drug-activated channels in various muscle membranes (Neher & Sakmann, 1976; Neher, Sakmann & Steinbach, 1978; Patlak, Gration & Usherwood, 1979).

One limitation of the patch-clamp approach has been that the concentration of agonist used to activate channels cannot be readily altered whilst recording. Here we describe a method for the internal perfusion of patch electrodes and use both conventional and internally perfused patch electrodes to examine parameters of glutamate- and agonist-activated channels in the extrajunctional membrane of locust muscle fibres. A preliminary report describing the perfusion system has appeared previously (Cull-Candy, Miledi & Parker, 1981).

METHODS

Preparation and solutions. The extensor tibiae muscle of the metathoracic leg of the locust (*Schistocerca gregaria*) was used throughout these experiments (Hoyle, 1955). The muscle was dissected as previously described and viewed with transmitted light after removal of the cuticle which covered the dorsal and ventral aspects of the femur. Muscles were maintained in a normal locust bathing medium or a Cl^--free medium at pH 6·8 (Cull-Candy, 1976). The input resistance of muscle fibres was increased in Cl^--free medium. Muscles were treated for 15–30 min with 1 μM-concanavalin A (con A) and then washed with normal medium before use (Patlak *et al.* 1979).

Patch-clamp electrodes contained an agonist dissolved in bathing medium. Intracellular micro-electrodes were filled with 3 M-KCl or 2M-K acetate or citrate. In some experiments muscle fibres were voltage-clamped with a conventional two-point clamp. To ensure that the membrane potential of the area under the patch pipette was well controlled, voltage recording and clamping electrodes were inserted close to each other (usually $\sim$100–200 μm) and the patch-clamp electrode was positioned between the two intracellular micro-electrodes. Additionally, Cl^--free medium was used to improve the quality of the space clamp.

Patch-clamp recording and analysis. Patch-clamp recordings were obtained using a circuit and pipettes similar to those previously described (Neher *et al.* 1978). Pipettes with internal tip diameter of about 2 μm were generally used and had resistances of around 2 MΩ when filled with locust medium. Seals showing an increase in resistance of 3–7 times (seal factor) could be achieved by pressing the patch pipette against the side of a muscle fibre, even though the muscles were not enzyme treated to remove connective tissue. We were, however, unable to improve the seal resistance further than this and the data shown have been corrected for attenuation of the signals by the shunt resistance, by multiplying by $1 + 1/\text{seal factor}$.

Single-channel records were recorded on FM tape with a band width of DC-1 kHz, and were later digitized at a sampling interval of 0·5 msec for computer analysis. Analysis was performed by displaying blocks of 512 data points and setting a cursor by eye midway between the base line and channel open states. Histograms were then automatically constructed of channel amplitudes and open and closed times. Records from membrane patches which showed any simultaneous openings of two or more channels, during long (50 sec) recording periods, were rejected for analysis. These selected patches therefore almost certainly contained only one active channel. We estimate the probability of observing zero simultaneous openings in 50 sec if two active channels were present as e^{-200}, for a glutamate concentration of 100 μM. This was further substantiated in perfusion experiments, where higher glutamate concentrations were applied to the same patch and no double sized events were detected.

Perfusion of the patch electrode. To allow the concentration of agonist at the membrane patch to be changed, a multibarrel perfusion pipette was inserted inside the patch electrode so that different solutions could be injected by pressure into the tip of the electrode (Fig. 1 *A*). The perfusion pipette was pulled by hand from multibarrel electrode glass (Clark Electromedical). A capillary about 8 cm long and 500 μm diameter was first formed, and a final taper then pulled to give a tip diameter of less than 50 μm. Syringe needles were cemented into the back of the pipette barrels to facilitate their filling with locust medium containing various concentrations of agonsit. The patch pipette

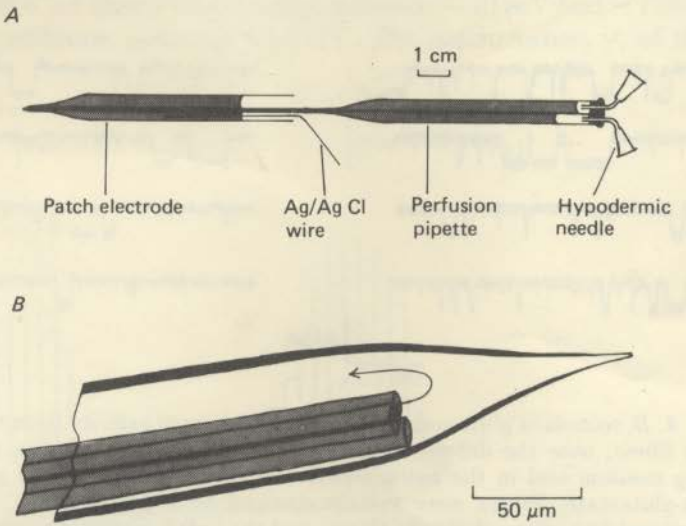

Fig. 1. *A*, diagram showing the arrangement of a perfusion pipette inserted in a patch electrode. For clarity the diameter of the pipettes has been exaggerated, and only two barrels are shown on the perfusion pipette. See text for details. *B*, tracing from a photomicrograph of the tip of a patch-clamp pipette containing an internal triple-barrel perfusion pipette. The arrow indicates the direction of flow of solution from a perfusion barrel during the application of pressure.

was filled initially with medium containing 100 μM-glutamate (this gave a convenient frequency of channel openings for locating good recording patches), and was mounted together with its internal perfusion pipette on a Zeiss double-electrode holder. The fine adjustments of the electrode holder allowed the tip of the perfusion pipette to be positioned to within about 100 μm of the patch-electrode tip (Fig. 1 *B*). A chlorided silver wire, for recording, was inserted in the patch pipette alongside the perfusion capillary. Flexible plastic tubes were connected to the syringe needles cemented in the perfusion barrels, and air pressure from a syringe was used to expel fluid from any desired barrel. Some drug leakage occurs from barrels which are not pressurized, and so a steady pressure was maintained on the desired barrel during recording, so that this solution would be in considerable excess in the patch-pipette tip. The reservoir of solution in the perfusion pipette was sufficient to allow several minutes' recording with each solution. We have successfully used perfusion pipettes containing up to six barrels.

RESULTS

Single-channel recordings in concanavalin A treated and untreated muscle

A low frequency of square-pulse currents is obtained when a patch electrode containing glutamate (> 100 μM) is applied to an extrajunctional site in untreated normal locust muscle fibres. Since the events are not observed when glutamate is removed from the patch electrode they presumably result from the activation of

glutamate-sensitive extrajunctional D-receptors which occur in normal innervated locust muscle fibres (Cull-Candy, 1976) and cause an inward current flux. To increase the frequency with which single-current events can be recorded the muscles were pre-treated with con A (see Patlak *et al.* 1979), which reduces desenitization of these receptors (Mathers & Usherwood, 1977). Most of the experiments which are described subsequently were performed after treatment with con A. Fig. 2 shows examples of

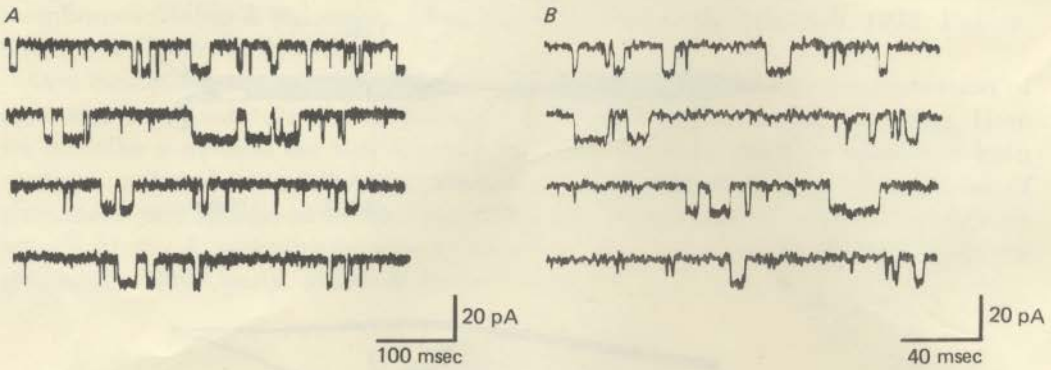

Fig. 2. *A*, *B*, records of glutamate-induced single-channel activity from two con A treated muscle fibres; note the difference in sweep speeds. Cl^--free solution was used for the bathing medium and in the patch pipette; in both experiments the pipette contained 100 μM-glutamate. Fibres were voltage-clamped to a potential of -110 mV with a conventional two-micro-electrode clamp, and the patch pipette was placed between the recording and current-passing electrodes. Recording band width was DC to 1 kHz. Inward current is shown as a downward deflexion. $T = 22$ °C, calibration 100 or 40 msec and 20 pA.

single-channel currents obtained with a patch clamp from two treated muscle fibres voltage-clamped at -110 mV. Following treatment there was a marked increase in the frequency of single-channel currents. Even when the frequency of channels was high only a low proportion of double events was seen at this membrane patch, which may indicate that the number of active receptors occurring under the pipette was small. Generally, recording sites were selected where there appeared to be only one active channel under the pipette. In treated and untreated preparations some muscle fibres showed no apparent activity at any site, even after several minutes of observation. If activity was found on a fibre it would usually be found at some other sites tried. These differences may reflect a difference in the density of extrajunctional receptors among the different fibres within a muscle.

Voltage dependence of single-channel current amplitude

Fig. 3*A* and *B* shows amplitude histograms of single-channel currents from a cell voltage-clamped at -50 mV and -110 mV. The single-channel current amplitudes show an approximately Gaussian distribution which is shifted to higher values as the cell is hyperpolarized. In this fibre the mean value for the single channel current, i, increased from $i = 5{\cdot}7$ pA at -50 mV to $i = 10{\cdot}4$ pA at -110 mV.

Amplitude histograms were sometimes bimodal, with a population of smaller channels being present. This probably resulted from channels located under the rim

of the patch pipette as described for acetylcholine (ACh) activated channels (Neher *et al.* 1978). Records from such membrane patches were rejected for analysis. Also, channel openings of 1 msec duration or shorter were not included for calculating mean amplitudes, since the restricted frequency response of the recording system attenuated these events. Fig. 3*C* illustrates the relationship between single-channel current and clamp potential. In most fibres examined the channel current was linearly dependent on clamp potential (i.e. an ohmic relationship) between −40 mV and −120 mV with an extrapolated equilibrium potential of 0 mV. The conductance, γ, of the single

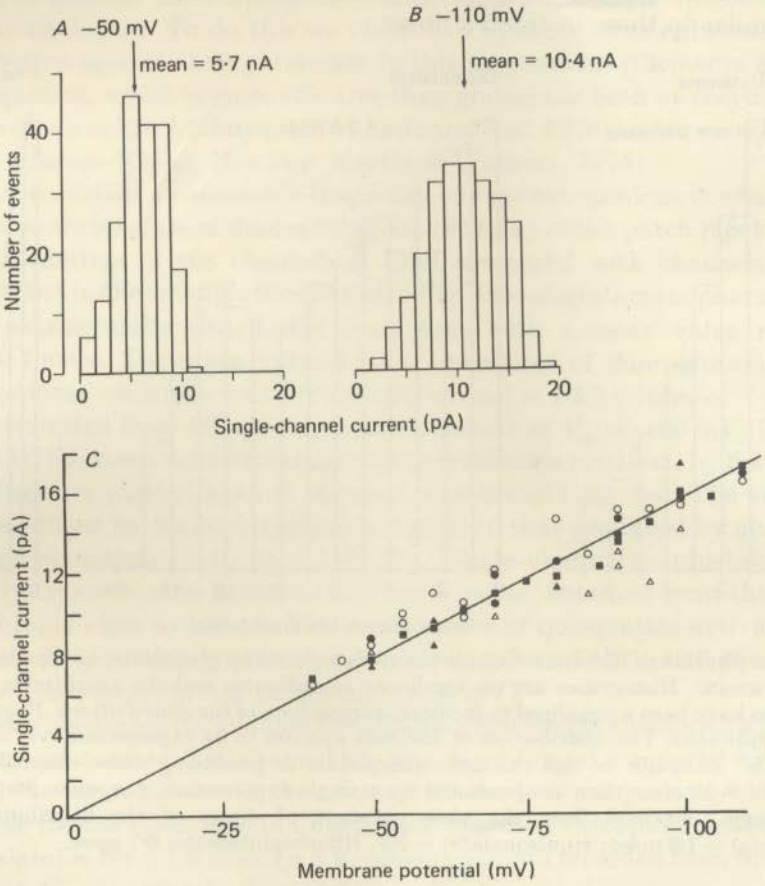

Fig. 3. Dependence of single-channel current on membrane potential. *A* and *B*, amplitude distribution of single-channel currents recorded from the same patch at two membrane potentials. Data are from all channel openings detected (including very brief events). *A*, $V_m = -50$ mV, mean amplitude (indicated by arrow) of single-channel current = 5·7 ± 0·15 pA (±S.E. for 187 events). *B*, $V_m = -110$ mV, mean amplitudes of single-channel current = 10·4 ± 0·02 pA (±S.E. for 239 events). *C*, mean single-channel current as a function of membrane potential. Each point is the mean value of approximately 100–200 events. Channel openings of less than 1 msec were not included in calculating the mean. Filled symbols are mean currents for glutamate-induced channels obtained from three muscle fibres. Open symbols are mean currents for fluoroglutamate-induced channels obtained from two muscle fibres. The regression line fitted by the least squares method to the glutamate data passes through 0 mV. The slope of the regression line corresponds to a single-channel conductance of 150 pS.

channel estimated in three muscle fibres, in which recording conditions were particularly good, was 150 pS over the entire range of membrane potentials (Fig. 3*C*).

In a few fibres the amplitude of the current was not linearly related to clamp potential but increased less than expected, with membrane hyperpolarization. At present it is uncertain whether this may be accounted for by inadequate clamp control, or whether the glutamate-activated channels in some fibres show rectification. These possibilities are being further examined. In fibres which had not been treated with con A, channel openings of low frequency (about 1 per min) could sometimes be observed. Although not systematically studied the amplitude of these currents appeared similar to those in treated fibres.

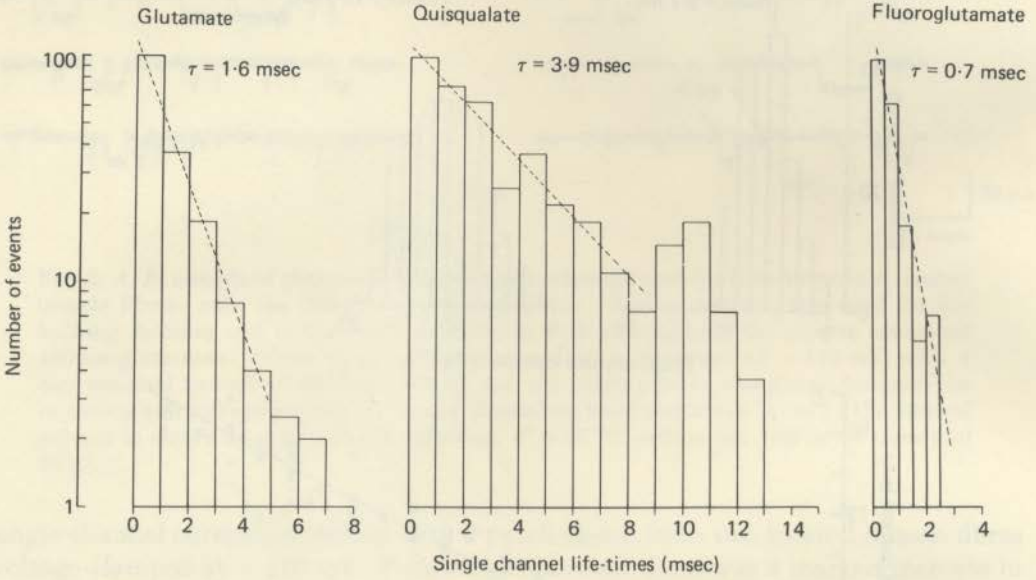

Fig. 4. Distribution of lifetimes of single channels induced by glutamate, quisqualate and fluoroglutamate. Histograms are on log–linear co-ordinates and the amplitudes of the histograms have been normalized to facilitate comparison of the distributions. Regression lines are indicated. The distribution of lifetimes appears to be exponential over at least one decade; although in this example quisqualate is producing more channels with lifetimes of 9–12 msec than are predicted by a single exponential. The mean lifetime of the channels, obtained from the time constant of decay of the distribution, is τ(glutamate) = 1·6 msec; τ(quisqualate) = 3·9; τ(fluoroglutamate) 0·7 msec.

Single-channel lifetimes

The lifetimes of channels induced by glutamate generally show an exponential distribution with a semi-logarithmic plot fitting well to a straight line (Fig. 4). Estimates of the mean channel lifetime were usually obtained from the slope of this line, since unlike the over-all mean value, this estimate is unaffected by the loss of very brief events due to filtering by the recording system.

A complication in obtaining channel lifetimes is that single-channel recordings sometimes show spontaneous and marked changes in their mean open time. In agreement with previous observations (Patlak *et al.* 1979) we find that records may show periods of several seconds duration during which the mean lifetime is two to

three times longer than normal and the frequency of channel openings is increased. We therefore restricted analysis of channel lifetimes to segments of records where channel behaviour was 'normal', the distribution of lifetimes displaying a single exponential. The mean open time of glutamate-operated channels was τ(glutamate) = 2·3 ± 0·12 msec (± S.E., single channels recorded from seventy two membrane patches), V_m = 60 mV, T = 23 °C

Single channels induced by agonists

It was of interest to compare the characteristics of single channels opened by glutamate analogues. To do this we chose two agonists: fluoroglutamate, which is a less effective agonist than glutamate in this preparation (Clements & May, 1974) and quisqualate, which is more effective than glutamate both at crayfish and locust junctions (Shinozaki & Shibuya, 1974; Anderson *et al.* 1976) and in mammalian spinal neurones (Biscoe, Evans, Headley, Martin & Watkins, 1975).

In order to obtain a reasonable frequency of channel openings it was necessary to use a high concentration of fluoroglutamate (500 μM) in the patch pipette (see later). The mean lifetime of the channels is brief compared with channels induced by glutamate and in the example illustrated in Fig. 4 fluoroglutamate produced channels with an exponentially distributed open time with a mean value τ(fluoroglutamate) = 0·7 msec. The mean value for the open time of fluoroglutamate-operated extra-junctional channels was τ(fluoroglutamate) = 1·4 ± 0·10 msec (± S.E.; single channels recorded from fifteen membrane patches) at V_m = −60 mV, T = 23 °C.

In Fig. 3*C* the mean values for single-channel currents produced by fluoroglutamate in two fibres are plotted against clamped membrane potential. The single-channel current produced by fluoroglutamate is similar to that produced by glutamate over a range of potentials (−40 to −110 mV). Single–channel conductance, γ, is not apparently dependent on potential and has a value, obtained from the slope of the relationship, of close to 150 pS. The conductance of quisqualate-activated channels was examined at resting potential, and a mean value of 137 ± 10·6 pS was obtained from four fibres (equilibrium potential = zero mV).

Fig. 4 also illustrates the channel lifetime distribution obtained with a patch pipette containing quisqualate. The channel life time is prolonged compared with glutamate channels; the mean lifetime in the example shown is τ(quisqualate) = 3·9 msec. The mean value for the open time of quisqualate operated extrajunctional channels was τ(quisqualate) = 6·4 ± 1·0 msec (± S.E., single channels recorded from four membrane patches) at V_m = −60 mV, T = 23 °C.

Voltage sensitivity of glutamate-induced channel lifetime

From noise analysis, the mean lifetime of glutamate-induced channels at the junctional membrane of locust muscle fibres has been shown to decrease as the membrane is hyperpolarized (Anderson *et al.* 1976). In contrast, we find that the mean single-channel lifetime recorded from con A treated muscle does not show any significant voltage sensitivity. Fig. 5 shows pooled data from nine muscle fibres, recorded using patch electrodes containing glutamate. There was no significant voltage sensitivity at potentials between −50 and −110 mV and, although there is considerable scatter in the data, a change in open time with the magnitude previously

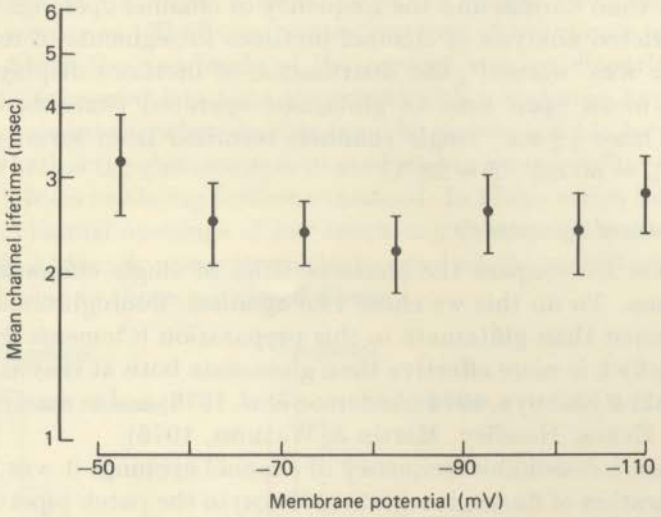

Fig. 5. Lifetime of glutamate-induced channels, plotted against membrane potential. Each point is the mean value of individual runs of 100–200 events from seven to nine fibres. Bars indicate ±1 S.E. of mean. Data are from con A treated muscles, $T = 20–25$ °C.

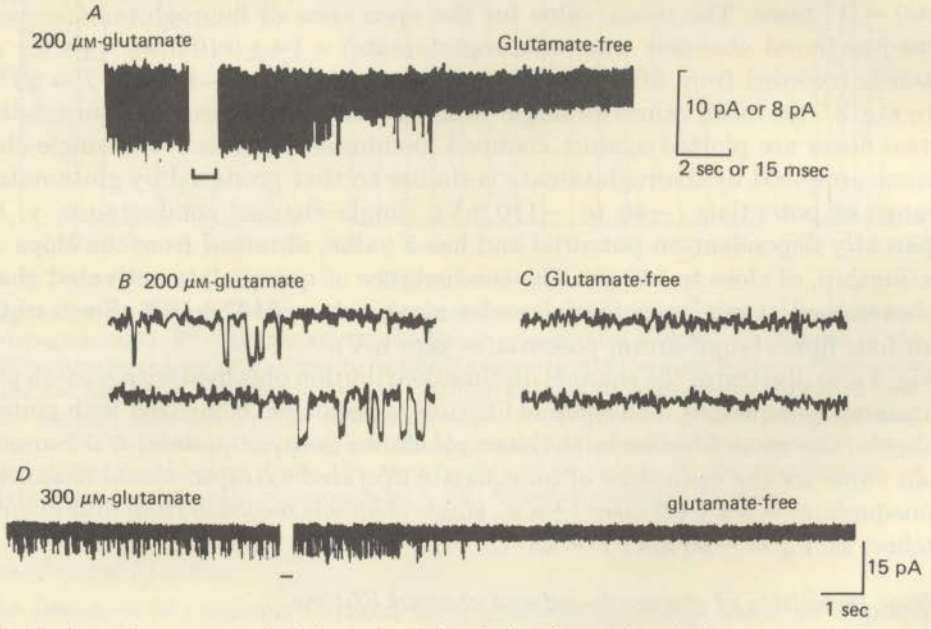

Fig. 6. *A*, continuous record of channel activity during internal perfusion of a patch pipette with 200 μM-glutamate followed by glutamate-free solution. The change in air pressure applied to the two perfusing barrels was made at the time indicated by the bar. Exchanging solutions was usually marked by an artifact (blanked out). Channel activity ceased about 8 sec after internal perfusion with glutamate-free solution had started. *B*, single-channel currents (on a fast sweep) recorded in the presence of 200 μM-glutamate (during the early section of the run illustrated in *A*). *C*, absence of channels in glutamate-free solution recorded during a late section of the run. Traces in *A*, *B* and *C* were all obtained during the same continuous run at a single site (analogue tape playback). Since no double sized events were seen at this site, it seems only one active channel was present. Calibration, 10 pA, 2 sec (*A*) or 8 pA, 15 msec (*B* and *C*). *D*, continuous record of channel activity (different fibre from *A*) during internal perfusion of a pipette with 300 μM-glutamate followed by glutamate-free medium. Channel activity disappeared about 5 sec after perfusing the electrode with glutamate-free solution.

described (Anderson, Cull-Candy & Miledi, 1978) should have been detectable. These results therefore support earlier findings (Dudel, 1979; Mathers, 1981) in suggesting that con A abolishes the voltage sensitivity of glutamate–activated channels.

Perfusion of the patch electrode

The perfusion system described in the Methods allowed changes in drug solutions to be made while recording from a single site, as illustrated in Fig. 6*A* and *D*. In the initial part of the records the patch pipette was perfused with 200 μM-glutamate and a high frequency of single-channel openings was present, as is seen more clearly when the records are examined on a faster sweep (Fig. 6*B* and *C*). The 200 μM-glutamate was exchanged for glutamate–free medium at the time indicated (bars in Fig. 6*A* and *D*) and events disappeared completely within about 8 sec. It was usually possible to change to a fresh medium within a few seconds, although when careful measurements were being made in the presence of the new solution longer periods (usually 20–30 sec) were allowed to ensure a more complete exchange of fluid in the pipette tip.

Distribution of channel open times and closed times

Fig. 7*A* and *D* shows examples of single-channel currents obtained in the presence of 100 and 200 μM-glutamate applied to a single extrajunctional site with an internally perfused patch electrode. The frequency of channel openings is markedly increased at the higher concentration. Histograms of channel open and closed times were obtained from measurements of several hundred openings obtained in 100 μM-(Fig. 7*B* and *C*) and 200 μM-glutamate (Fig. 7*E* and *F*). The distribution of open and closed times can be fitted with single exponentials and in this example the mean channel lifetime was 1·0 msec in 100 μM-glutamate and 0·9 msec in 200 μM-glutamate. The mean channel closed time decreased from 21 msec in 100 μM-glutamate to 5·6 msec in 200 μM-glutamate.

Dependence of channel closed time on glutamate concentration

Using internally perfused patch electrodes it was possible to examine the 'dose–response' characteristic of individual receptor–channel complexes by applying various concentrations of glutamate to sites where only a single channel appeared active. Thus no double sized events were present even at very high glutamate levels (e.g. 600 μM), when the channel was seen to be in the open state for a majority of the time. Fig. 8*A* illustrates typical data from one experiment in which the inverse of the mean channel closed time, $1/\tau_{\text{closed}}$, is plotted against glutamate concentration (filled symbols). The data were obtained from four different membrane patches in different fibres, and between two and five concentrations of glutamate (varying between 50 and 600 μM) were applied to individual patches by internal perfusion of the patch electrode. Records were obtained at resting membrane potential (*ca.* −60 mV). The continuous line fitted to the mean values of $1/\tau_{\text{closed}}$ has a slope value of 2·1 on double logarithmic co-ordinates. This relationship illustrates the dependence of the apparent net forward reaction rate on glutamate concentration.

The mean open time of the channels was also estimated from the same records, and the reciprocals of the mean open times $1/\tau_{\text{open}}$ are indicated by open symbols. At high glutamate concentrations the open time distributions became distorted

because many channel closings were so brief as to be undetectable by the recording system. In these cases a reasonable estimate of the channel open time could still be made from the initial exponential part of the life time distribution. The mean open life time obtained in this way did not appear to be dependent upon glutamate concentration over the range of values explored, and the dashed line in Fig. 8*A* is

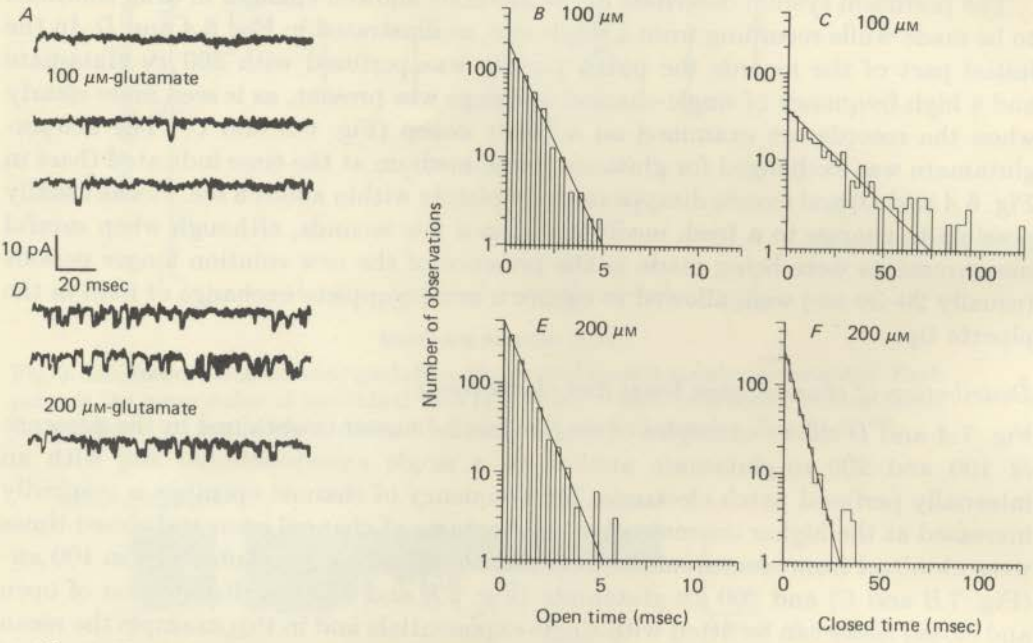

Fig. 7. Examples of channel openings recorded in the presence of two different glutamate concentrations applied to the same membrane patch. *A*, *B* and *C*, traces and corresponding histograms of events obtained with a patch electrode perfused with 100 μM-glutamate. *D*, *E* and *F*, patch electrode perfused with 200 μM-glutamate. At each concentration the traces are samples from recordings lasting several minutes (analogue tape playback). At this patch only one channel appeared active: no double sized events were seen even with high glutamate concentrations (600 μM) (not shown). *B* and *E*, distribution of channel open times for two concentrations can be well fitted with straight lines (on semilogrithmic co-ordinates), indicating an exponential distribution. Lines fitted to the data correspond to a mean open time of 1 msec in 100 μM-glutamate and 0·9 msec in 200 μM-glutamate. *C* and *F*, distribution of channel closed times. Straight lines fitted to the data correspond to a mean channel closed time of 21 msec in 100 μM-glutamate and 5·6 msec in 200 μM-glutamate. Calibration, 10 pA, 20 msec; $T = 22$ °*C*.

drawn through the mean value of $1/\tau_{open}$. In this experiment the mean channel life time was only about 1 msec, probably because the temperature ($T = 28$ °C) was higher than usual. The intersection of the dashed and continuous line in Fig. 8*A* indicates the glutamate concentration at which the channels are in the open state for 50% of the time. In these fibres the mean open and closed times are equivalent at a glutamate concentration of 500 μM (indicated by arrow in Fig. 8*A*). This therefore represents the apparent dissociation constant for channel activation by glutamate.

Fig. 8*B* shows pooled data of $1/\tau_{closed}$ measured from seventeen membrane patches, plotted as in Fig. 8*A*. The slope of the regression line fitted to the data is

1·85 on double logarithmic co-ordinates. We estimate that the mean lifetime of glutamate-activated channels is approximately 2·0 msec in these fibres at room temperature, which is similar to the values of about 2·5 msec obtained with noise analysis from untreated junctional receptors at a clamp potential of −80 mV (Anderson *et al.* 1978). With a lifetime of 2–2·5 msec the apparent dissociation constant for channel activation by glutamate is about 300 μM from the relationship in Fig. 8*B*.

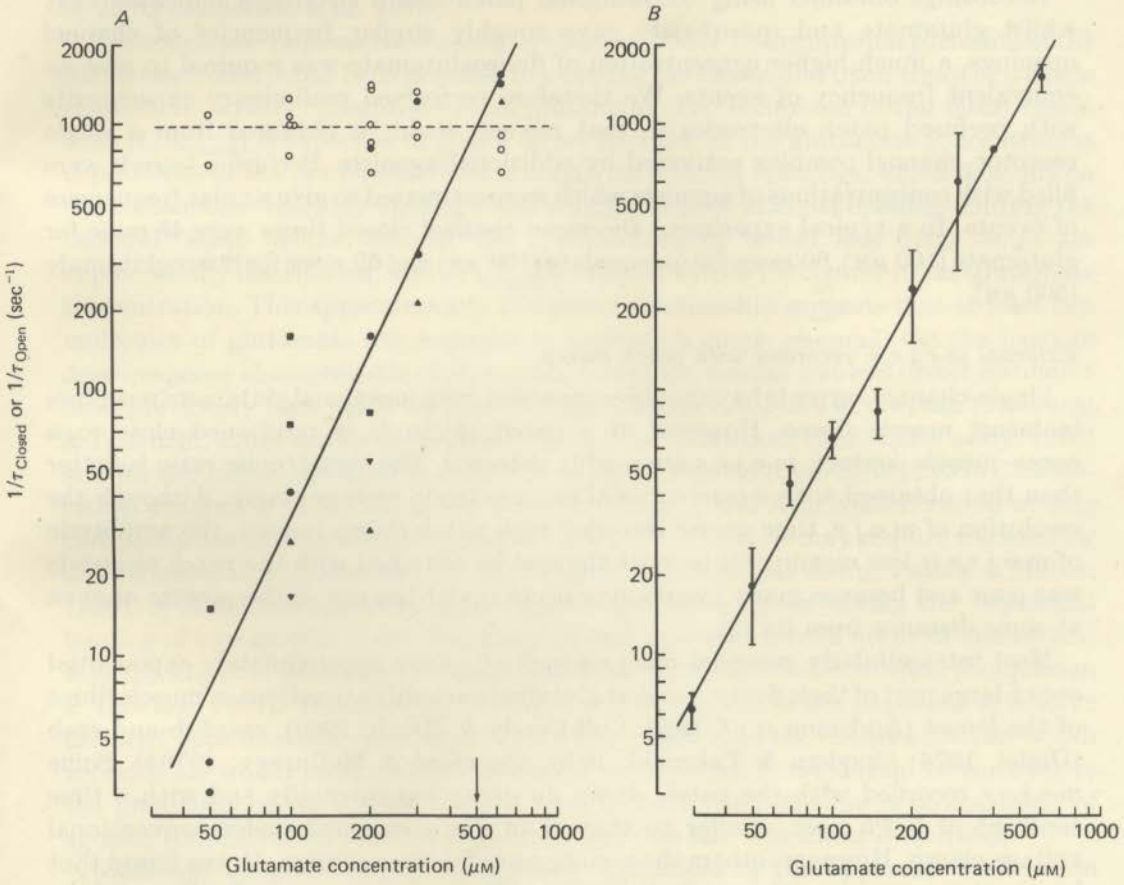

Fig. 8. Relationship between the reciprocal of mean channel closed time, $1/\tau_{closed}$, and glutamate concentration. *A*, $1/\tau_{closed}$ (filled symbols) and the reciprocal of the mean channel open time, $1/\tau_{open}$, (open circles) are plotted against the concentration of glutamate contained in the patch pipette (logarithmic co-ordinates). Measurements of $1/\tau_{closed}$ were obtained from four membrane patches in different muscle fibres(●, ■, ▲, ▼). Several glutamate concentrations were tested at each site, and $1/\tau_{closed}$ was estimated from the time constant of the distribution histogram (as shown in Fig. 7 *C* and *F*). The continuous line through the points is fitted by the least squares method and has a slope of 2·1. The mean value of $1/\tau_{open}$ (○) obtained from the same experimental records is indicated by the dashed line. Open and closed times are equal at the intercept of the lines fitted to $1/\tau_{closed}$ and $1/\tau_{open}$, which occurs at a glutamate concentration of 500 μM (indicated by arrow). *B*, relationship constructed from estimates of $1/\tau_{closed}$ obtained at seventeen membrane patches. The continuous line through the points has a slope of 1·85. All values are mean ± 1 S.E. (except at 400 μM-glutamate which is only one value).

To decide whether there was any marked change in receptor affinity following con A treatment, minature excitatory junctional current amplitudes were measured. In a typical experiment, in an untreated preparation $\overline{\text{m.e.j.c.}} = 1{\cdot}54 \pm 0{\cdot}07$ nA (four fibres), in a treated preparation from the same animal $\overline{\text{m.e.j.c.}} = 1{\cdot}48 \pm 0{\cdot}13$ nA (five fibres), $V_m = -80$ mV and $T = 21$ °C. Thus no evidence was found for a change in the affinity of junctional receptors for neurally released transmitter.

Channel closed times for various agonists

Recordings obtained using conventional patch-clamp electrodes indicated that whilst glutamate and quisqualate gave roughly similar frequencies of channel openings, a much higher concentration of fluoroglutamate was required to give an equivalent frequency of events. We therefore performed preliminary experiments with perfused patch electrodes so that records could be obtained from a single receptor–channel complex activated by additional agonists. Perfusion barrels were filled with concentrations of agonists which were estimated to give similar frequencies of events. In a typical experiment the mean channel closed times were 48 msec for glutamate (100 μM), 60 msec for quisqualate (100 μM) and 69 msec for fluoroglutamate (500 μM).

External m.e.j.c.s. recorded with patch clamp

Single-channel currents have not been recorded from junctional glutamate receptors in locust muscle fibres. However, if a patch electrode is positioned close to a nerve–muscle contact, m.e.j.c.s are readily detected. The signal/noise ratio is better than that obtained with a conventional two-electrode voltage clamp. Although the resolution of m.e.j.c. time course recorded with patch clamp is good, the amplitude of m.e.j.c.s is less meaningful because the seal we obtained with the patch electrode was poor and because many events may occur under the rim of the pipette or even at some distance from its tip.

Most intracellularly recorded m.e.j.c.s and e.j.c.s are approximately exponential over a large part of their decay phase at glutamate-sensitive junctions in muscle fibres of the locust (Anderson *et al.* 1976; Cull-Candy & Miledi, 1980), crayfish and crab (Dudel, 1974; Onodera & Takeuchi, 1978; Crawford & McBurney, 1976*b*). Some m.e.j.c.s recorded with the patch clamp do decay exponentially and with a time constant of 2–2·5 msec, similar to that of m.e.j.c.s recorded under conventional voltage clamp. However, others show more complex time courses. It was found that by exerting pressure on the patch electrode, as fibres were mechanically distorted the proportion of m.e.j.c.s decaying non-exponentially was markedly increased and in some experiments virtually all events recorded had complex time course. A similar phenomenon has been observed at vertebrate end-plates afer inhibition of cholinesterase (Katz & Miledi, 1973*a*).

DISCUSSION

Single-channel parameters. The conductance, γ, of the single extrajunctional channels activated by glutamate is close to 150 pS when obtained with patch clamp, which is similar to the conductance of junctional channels obtained from noise

analysis (Anderson *et al.* 1978; Cull-Candy & Miledi, 1980) and of extrajunctional channels previously studied with patch clamp (Patlak *et al.* 1979). We found no evidence for marked changes in channel conductance following treatment with con A, although the voltage sensitivity of the channel lifetime was apparently abolished, as previously described for crayfish (Dudel, 1979) and locust muscle (Mathers, 1981). During most of the observation periods the lifetime of the extrajunctional channels showed an exponential distribution with a mean of 2·3 msec, similar to the lifetime of junctional channels obtained at a clamp potential of −60 mV and a temperature of 23 °C (Anderson *et al.* 1978).

Concentration–response relationship of single channels. Using internal perfusion of the patch electrodes it has been possible to examine the closed and open times of a single channel at various concentrations of glutamate. The reciprocal of the mean closed time ($1/\tau_{closed}$) is expected to give a direct measure of the glutamate concentration dependence of the net forward reaction rate for channel openings, which depends on the transmitter–receptor binding steps which preceded channel opening. During the 'normal' state of the channel the distributions of closed and open times are exponentially distributed, and $1/\tau_{closed}$ increased with a 1·85 power of the glutamate concentration. This approximately 2nd power relationship suggests that at least two molecules of glutamate are required to activate a single channel. On the basis of dose–response characteristic of glutamate receptors, similar but less direct estimates have previously been made for junctional glutamate receptors in crayfish (Takeuchi & Takeuchi, 1964) and locust muscle (Walther & Usherwood, 1972; Cull-Candy, 1978) and for the extrajunctional D-receptors in locust muscle (Cull-Candy, 1976). Patch-clamp analysis of the relationship between $1/\tau_{closed}$ and ACh concentration at frog end-plates has also led to the suggestion that ACh-receptors may require the binding of two transmitter molecules to open a single channel (Sakmann, Patlak & Neher, 1980). A simple co-operative model for channel opening, involving the sequential binding of two agonist molecules, predicts that openings should occur in bursts, and that the length of these bursts should increase with agonist concentration (Colquhoun & Hawkes, 1981). The time resolution of our recordings is not expected to resolve the brief gaps between openings within a burst, and thus the channel lifetime which we estimate would presumably correspond to the mean burst length. In contrast to the prediction of the model, we find that the observed lifetime is independent of agonist concentration (50–600 μM-glutamate) (see also Sakmann *et al.* 1980).

Glutamate receptors have a low affinity. Estimates of the apparent dissociation constant were obtained from the glutamate concentration at which the channels are in the open state for 50 % of the time. For channels in the 'normal' kinetic state this yielded values of about 300 μM-glutamate, whilst estimates made during periods when the channel lifetime was spontaneously prolonged gave appreciably lower values. The cause of the change in channel kinetics is at present unclear and no evidence for these prolonged channel lifetimes has been obtained from excitatory junctional currents or noise analysis. On the other hand the lifetime of the single channel in the 'normal' kinetic state corresponds well with estimates from excitatory junctional currents and glutamate current noise.

Judging by the lack of change of m.e.j.c. amplitude in con A treated muscles it appears that the affinity of the receptors was not altered by this treatment. Hence

the apparent dissociation constant for extrajunctional glutamate receptors that we obtain is about ten times larger than the values of about 20 μM estimated for extrajunctional ACh-receptors in vertebrate muscle fibres (Sakmann *et al.* 1980).

If, as may be expected, the affinity of junctional receptors for nerve-released transmitter is similarly low, then only a small fraction of the transmitter packet may be expected to bind to the post-synaptic receptors. At the locust excitatory junction a single packet of transmitter opens only about 250 ionic channels (Cull-Candy & Miledi, 1980), whereas at the vertebrate end-plate a single packet of ACh opens about 1500–2000 ionic channels (Katz & Miledi, 1972; Anderson & Stevens, 1973). It may therefore be that the number of transmitter molecules contained in a quantal packet is similar at the locust and vertebrate nerve–muscle junction, despite the different transmitters involved.

Diffusion of transmitter in the cleft. If a proportion, p, of the transmitter molecules released during discharge of a single transmitter packet binds to the receptors, then diffusion of the transmitter out of the synaptic cleft will be slowed down by a factor $1/(1-p)$ (Katz & Miledi, 1973*a*). At the locust nerve–muscle junction we estimate that only a small proportion of the transmitter packet attaches to the receptor; hence $1/(1-p)$ approaches 1 and diffusion of the transmitter will be almost unrestricted. If free diffusion in the cleft is rapid, compared with the channel lifetime, then $\tau_{e.j.c.}$ should approximate the mean channel lifetime even in the absence of a mechanism for removing transmitter. Indeed, blockers of glutamate uptake have little effect on the time course of transmitter action at this junction (Clark, Gration & Usherwood, 1980). A further clue concerning this may be obtained from the complex decays of some m.e.j.c.s recorded with the patch clamp. M.e.j.c.s with abnormal time courses become particularly noticeable where mechanical pressure is exerted, which seems to be analogous to the 'compression artifact' described at cholinesterase-blocked vertebrate end-plates (Katz & Miledi, 1973*a*). It is thought that when ACh is prevented from being hydrolysed by cholinesterase, mechanical distortion of the tissue (by the pipette), causes local obstructions in the diffusion path by which the transmitter escapes from the synaptic cleft. The observation that a 'compression artifact' can be readily produced at normal locust junctions tends to suggest that diffusion of the transmitter out of the cleft is important in terminating transmitter action and that enzymatic destruction or uptake of glutamate by glia and nerve cells which is known to occur (Faeder & Salpeter, 1970; Salpeter & Faeder, 1971) may be of secondary importance in this respect. Blockers of glutamate uptake prolong the time course of $\tau_{e.j.c.}$ at the crab nerve–muscle junction (Crawford & McBurney, 1977) and it would therefore be of interest to determine if glutamate receptors at this junction have a higher apparent affinity for the transmitter than do receptors in locust muscle fibres.

Activation of channels by different agonists. The conductances of channels opened by glutamate, quisqualate and fluoroglutamate are similar; hence differences in efficacy of the agonists must result from differences in kinetics of the channel openings. It is of interest that fluoroglutamate, which is reported to be less effective than glutamate at causing contraction in locust muscle (Clements & May, 1974), produces channels with a mean lifetime which is approximately one third as long as those produced by glutamate. Furthermore, the affinity of receptors for fluoroglutamate

is markedly less than for glutamate. These two factors are probably sufficient to account for the low efficacy of the agonist. On the other hand, quisqualate, which is a more effective agonist than glutamate in some systems (Shinozaki & Shibuya, 1974; Biscoe *et al.* 1975; Anderson *et al.* 1976), produced channels with a mean lifetime approximately twice as long as those produced by glutamate as previously demonstrated by analysis of noise and patch-clamp records (Anderson *et al.* 1976; Gration & Usherwood, 1980). However, the receptor affinity for quisqualate was similar to or slightly higher than the affinity for glutamate, so for this agonist the increased efficacy in locust muscle may be due mainly to an increase in the lifetime of the channels which are activated. It has previously been reported, both for ACh and glutamate receptors, that the mean lifetime of the channel is agonist-dependent (Katz & Miledi, 1973*b*; Colquhoun, Dionne, Steinbach & Stevens, 1975; Anderson *et al.* 1976; Crawford & McBurney, 1976*a*).

We thank Sir Bernard Katz for helpful discussion, and Mr Rico Miledi for help with computer programming. This work was supported by the Medical Research Council and the Royal Society of London.

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