J. Physiol. (1977), **273**, *pp.* 367–388
With 10 *text-figures*
Printed in Great Britain

PERMEABILITY OF THE POST-SYNAPTIC MEMBRANE OF AN EXCITATORY GLUTAMATE SYNAPSE TO SODIUM AND POTASSIUM

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(*Received 28 September 1976*)

SUMMARY

1. The changes in permeability of the post-synaptic membrane at the insect skeletal neuromuscular junction caused by the excitatory transmitter and L-glutamate have been studied using the voltage clamp technique.

2. The reversal potential (E_R) of the excitatory post-synaptic current and the glutamate current was +3 and +4 mV respectively.

3. E_R of the synaptic current did not change when external K was altered between 0 and 20 mM, but did show a small positive shift in 40 mM external K. Reducing external Na to 1–10 mM changes E_R by 12–18 mV. Reducing external Cl to zero caused no change in E_R.

4. It is proposed that the transmitter and L-glutamate cause an increase in permeability to Na and K, but not to Cl.

5. In normal saline, the ratio of the permeability increase to Na and K ($\Delta P_{Na}/\Delta P_K$) is 0·9.

6. The changes in E_R caused by altering external K were similar to those predicted by the Goldman–Hodgkin–Katz equation, assuming $\Delta P_{Na}/\Delta P_K$ stays constant.

7. The changes in E_R caused by alterations of external Na are much less than those predicted by the Goldman equation.

8. No glutamate current could be recorded in Na- and Ca-free saline either at the resting potential or at depolarized or hyperpolarized membrane potentials.

9. It is proposed that the outward K current is dependent upon the inward Na current, and that the increase in K permeability is abolished in zero external Na.

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INTRODUCTION

Transmitter substances usually act on the post-synaptic membrane by opening channels which have a high ionic selectivity. For example, it has been well established that inhibitory transmitters usually cause an increase in permeability to either K or Cl (Boistel & Fatt, 1958; Trautwein & Dudel, 1958; Furshpan & Potter, 1959; Kehoe, 1972), and good evidence has been presented that the excitatory transmitter at the vertebrate end-plate involves an increase in permeability to Na and K, but not Cl (Takeuchi & Takeuchi, 1960; Takeuchi, 1963*a*, *b*). However, the ionic permeability changes at non-cholinergic excitatory synapses have not been investigated in detail.

There has been increasing evidence in recent years that L-glutamate is an excitatory transmitter in the vertebrate central nervous system and at the arthropod neuromuscular junction (Gerschenfeld, 1973; Krnjevic 1974), and L-glutamate has been shown to cause a depolarization and a conductance increase at the insect and crustacean neuromuscular junction (Takeuchi & Takeuchi, 1964; Usherwood & Machili, 1968; Gerschenfeld, 1973). Although there is evidence that this depolarization involves an increase in conductance to sodium ions (Onodera & Takeuchi, 1975; Takeuchi & Onodera, 1973), the extent to which other ions are involved is unclear. In this study, the voltage clamp technique has been used to make a detailed study of the ionic basis of the conductance increase caused by L-glutamate and the excitatory transmitter at the insect neuromuscular junction.

METHODS

Metathoracic legs from adult locusts (*Schistocerca gregaria*) were isolated at the trochanteral-coxal margin and placed in a Perspex perfusion chamber with a total volume of 2·5 ml. The extensor tibiae muscle was exposed along its length by removing the other muscles in the femur and was perfused with standard locust saline (see below).

The distal fibres of the extensor tibiae muscle were used in this study. These fibres have a diameter of about 100 μm and are about 1 mm in length. They are particularly suitable for voltage clamp studies by virtue of their long length constant, which was found to vary between 3·0 mm and 7·0 mm. In fact, when two recording electrodes were placed at either end of the muscle fibre, no difference in the amplitude of miniature excitatory post-synaptic potentials (m.e.p.p.s.) could be observed in most fibres. The voltage clamp technique using intracellular micro-electrodes, which was developed by Takeuchi & Takeuchi (1959) for studies on vertebrate end-plate, was used (Fig. 1). The efficiency of the clamp, measured at either end of a muscle fibre with a second recording electrode, was greater than 95 %. The potential recording and current injection micro-electrodes were filled with 2M-K citrate. High resistance electrodes (> 100 MΩ) for glutamate iontophoresis were filled with 1 M-Na L-glutamate at pH 7·5.

The normal saline had the following composition (mM): NaCl 170·0, KCl 10·0,

$CaCl_2$ 2·0, Hepes buffer 10·0, pH 7·0. In the ion substitution experiments, Na was replaced by choline and Cl by methyl sulphate. Increases in external K were made keeping the product of external KCl constant. All salines were buffered at pH 7·0. Preliminary accounts of some of the results have been described previously (Anwyl & Usherwood, 1974, 1975).

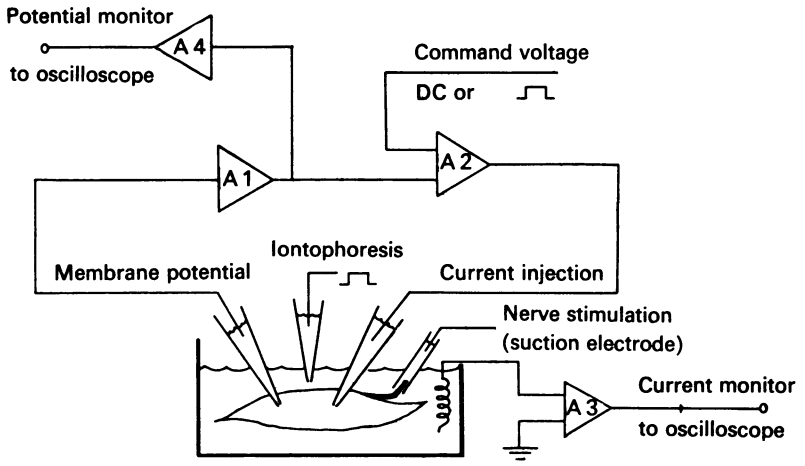

Fig. 1. Schematic diagram of the experimental apparatus. The membrane potential was recorded with an intracellular micro-electrode (5–10 MΩ resistance) and a high input impedance amplifier (A 1). A 2 is the voltage clamp control amplifier which was a high voltage operational amplifier (Philbrick 1022). Current was fed into the muscle fibre through a low resistance micro-electrode (4–6 MΩ). The current was measured with A 3 (Analog Devices 141B), used as a current to voltage converter, with the bath maintained at virtual earth.

RESULTS

The characteristic response to glutamate and to the natural transmitter

The locust extensor tibiae muscle is innervated by two excitatory motor neurones and one inhibitory motor neurone (Usherwood & Grundfest, 1965). The distal fibres are innervated by a 'fast' excitatory axon and some fibres also receive endings from a 'slow' excitatory axon. Stimulation of the 'fast' axon normally produced a large excitatory post-synaptic potential (e.p.s.p.) of 15–20 mV amplitude which was accompanied by an electrically excited spike-like response of the non-synaptic muscle membrane. Stimulation of the 'slow' axon resulted in a much smaller e.p.s.p. which did not activate the electrically excitable membrane of the muscle fibres. A few of the distal fibres are also innervated by the inhibitory neurone which supplies the extensor tibiae muscle.

The amplitude of the fast e.p.s.p. was reduced below the threshold for

activation of the action potential by using normal saline containing 40–50 mM-$MgCl_2$. The e.p.s.p has a rise time of 8–10 msec and a half-decay time of 40–60 msec (Fig. 2, trace *a*). When the muscle fibre was clamped at the resting potential, stimulation of the 'fast' axon caused a transient flow of inward current, the excitatory post-synaptic current (e.p.s.c.). The e.p.s.c. had a maximum amplitude of 1×10^{-6} A, a rise time of 1·8–2·5 msec and a half-decay time of 1·9–2·3 msec (Fig. 2, trace *b*).

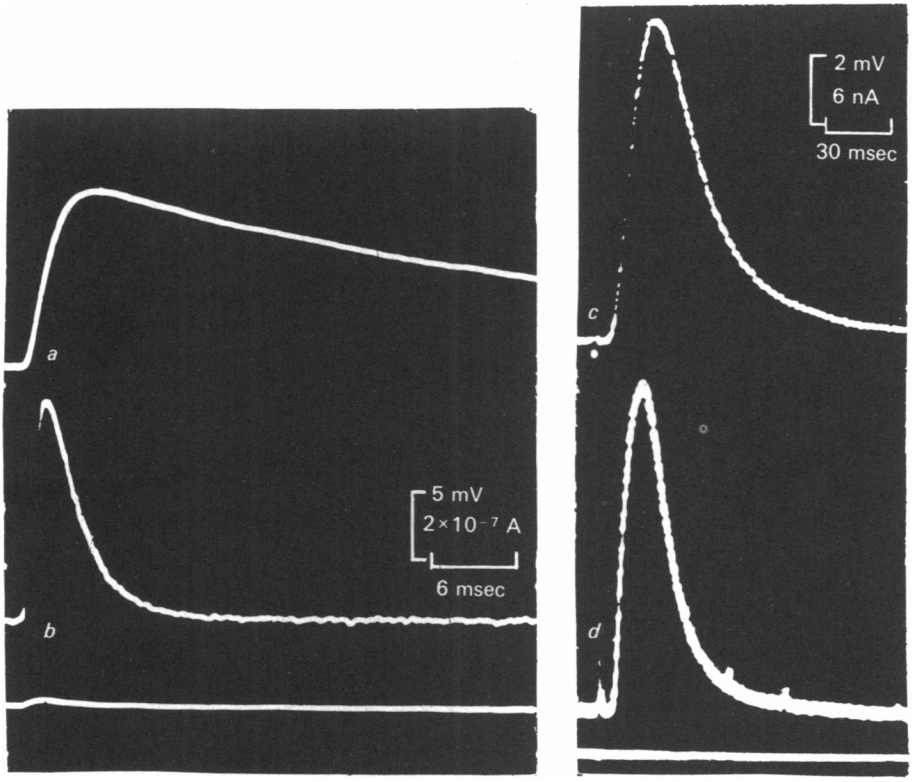

Fig. 2. Trace *a* shows the excitatory post-synaptic potential (e.p.s.p.) recorded intracellularly at the resting potential from an extensor tibiae muscle fibre. Top trace *b* shows an excitatory post-junctional current (e.p.s.c.) recorded from the same muscle fibre with the membrane clamped at the resting potential. Lower trace *b* is the membrane potential in the clamped muscle fibre. Only a very small e.p.s.p. occurs when the fibre is clamped. Inward current is shown as an upward deflexion. Trace *c*, glutamate potential recorded intracellularly at the resting potential. Trace *d*, upper trace shows the glutamate current recorded from the same synaptic site as in *c* with the membrane clamped at the resting potential. Lower trace shows the membrane potential in the clamped muscle fibre.

Iontophoresis of L-glutamate on to an excitatory junction produced a transient depolarization, the glutamate potential (Fig. 2, trace *c*). The rise time of the glutamate potential decreased as the drug electrode was moved

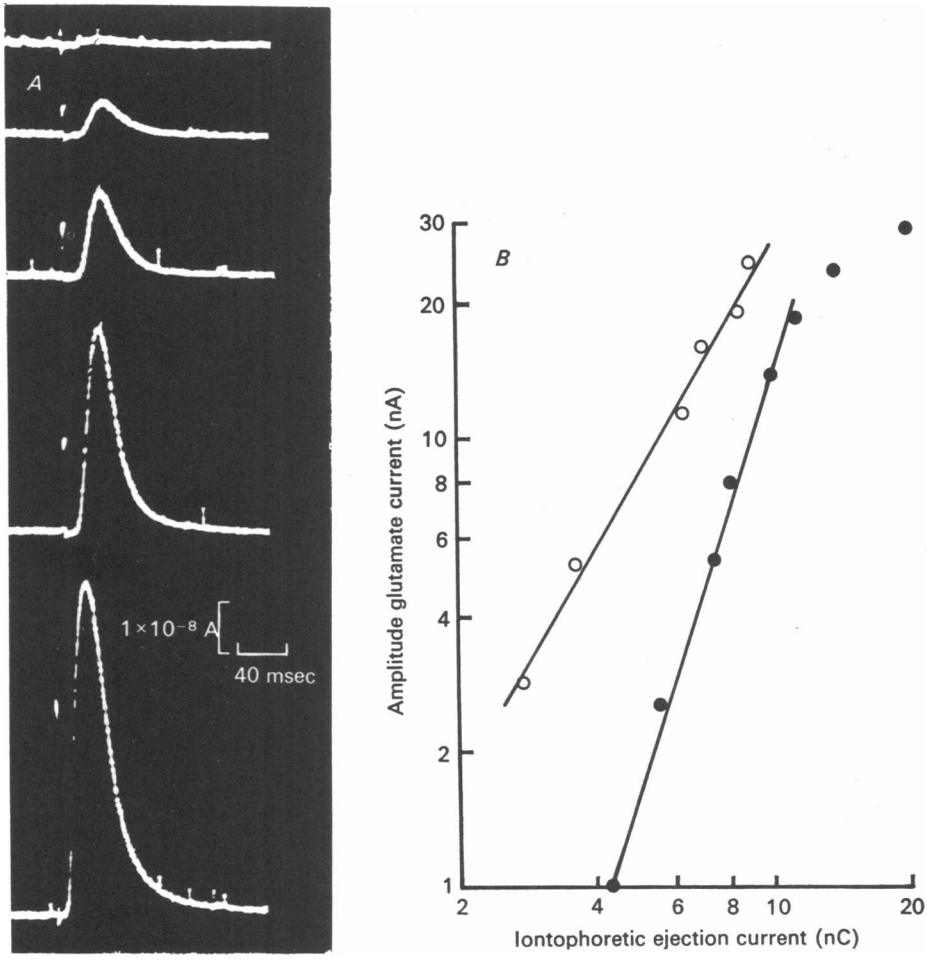

Fig. 3. *A*, relationship between amplitude of glutamate current recorded at a single junction of a superficial muscle fibre, and the amount of glutamate ejected from the iontophoretic electrode. *B*, dose–response for glutamate currents recorded at two neuromuscular junctions. Lines (drawn by eye) give estimates of maximum slopes of curves. These estimates are 1·75 (○) and 3·2 (●)

closer to the junctional site. Occasionally, glutamate potentials with a rise time of 10 msec were recorded, which is similar to the rise time of the e.p.s.p. Usually, however, the rise times of the glutamate potentials were much

longer than this, i.e. 20–50 msec. With the muscle fibre clamped at its resting potential an inward current was recorded during glutamate iontophoresis (Fig. 2, trace *d*). Glutamate currents of fast rise time (less than 20 msec) had a somewhat faster time course than the corresponding glutamate potential. With glutamate currents of slower rise time there was little difference in the time course of the glutamate potential and glutamate current. At a glutamate sensitive site, the amplitude of the glutamate current increased with increasing injection currents (Fig. 3*A*). Log-log plots of the relationship between the amplitude of the glutamate current and the ejecting current gave limiting slopes of between 1·8 and 3·0 (Fig. 3).

Iontophoresis of glutamate on to extrajunctional membrane of the locust muscle fibre caused a small transient depolarization followed by a transient but longer-lasting hyperpolarization (Cull-Candy & Usherwood, 1973). These responses, which rarely exceeded 2 mV in amplitude, are considered to have resulted from glutamate activation of two populations of extrajunctional receptors for this amino acid.

Reversal potentials for junctional currents

The amplitudes of the glutamate current and e.p.s.c. were determined at different clamped membrane potentials (Figs. 4, 5). When the muscle fibre was depolarized the inward currents first became smaller in amplitude and then reversed to become outward currents. The current–voltage relationship between the amplitude of either the glutamate current or the e.p.s.c. and the membrane potential was approximately linear between -60 and $+20$ mV (Figs. 4*B*, 5*C*). The reversal potential values for the glutamate current and the e.p.s.c. were very similar, i.e. the reversal potential for the glutamate current ($E_{g.c.}$) was $+4 \pm 5$ mV (mean $\pm$ S.D., $n = 15$), and the reversal potential for the e.p.s.c. ($E_{e.p.s.c.}$) was $+3 \pm 3$ mV, $n = 12$. In some experiments it was not possible to reverse the glutamate current and e.p.s.c. without causing contraction. However, extrapolated values for reversal potentials for such fibres were not significantly different from the values obtained from actual reversals.

Although iontophoresis of L-glutamate on to a junctional site seemingly produced only an inward current when the fibre was clamped at the resting potential, a biphasic current was often observed when the membrane potential was held at values more positive than this (Fig. 5*A*). This biphasic current consisted of an inward component of short duration followed by a small slower outward current. The outward current component of the biphasic glutamate current was abolished in chloride free saline (Fig. 5*B*) and was almost certainly caused by glutamate activation of hyperpolarizing extrajunctional receptors.

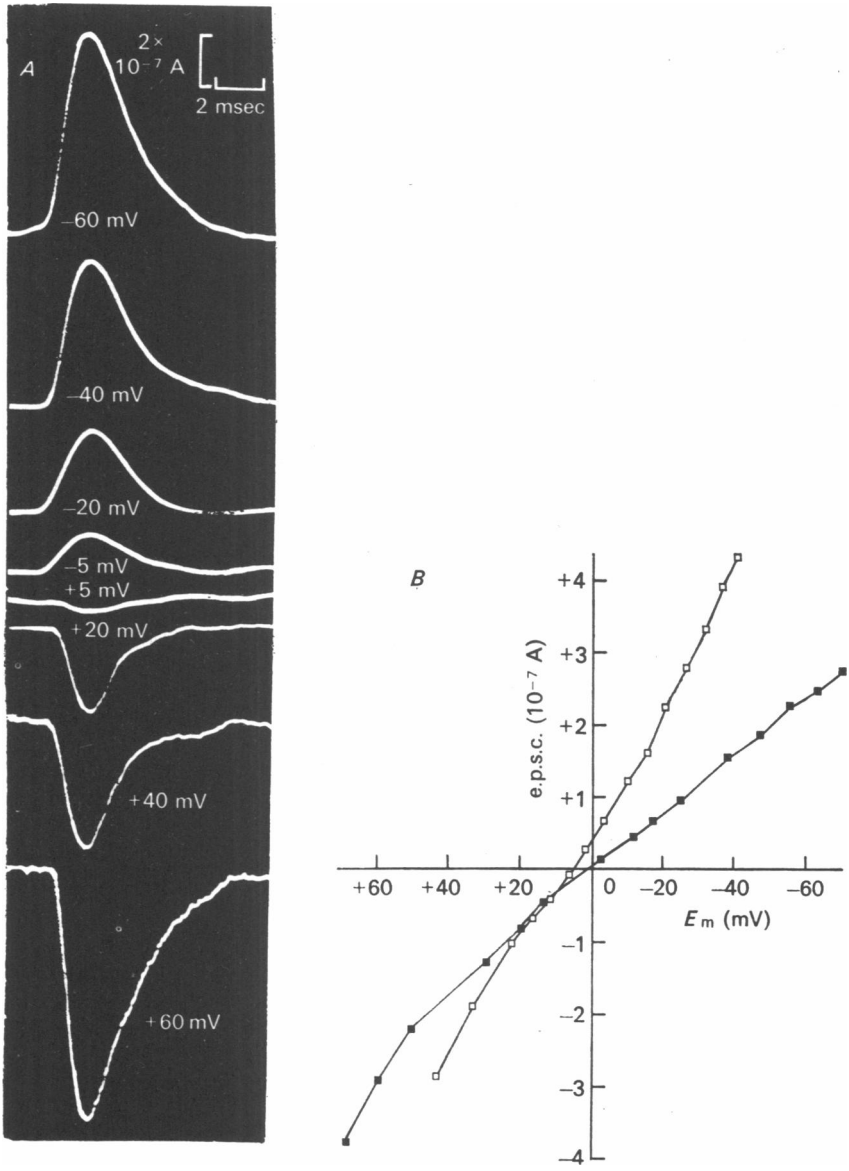

Fig. 4. *A*, e.p.s.c.s recorded at different clamped membrane potentials (shown in left-hand column). The reversal potential of the e.p.s.c. was about +2 mV. *B*, relationship between the membrane potential and the amplitude of two e.p.s.c.s (□ and ■) in normal saline. +ve, denotes inward currents and −ve, outward currents.

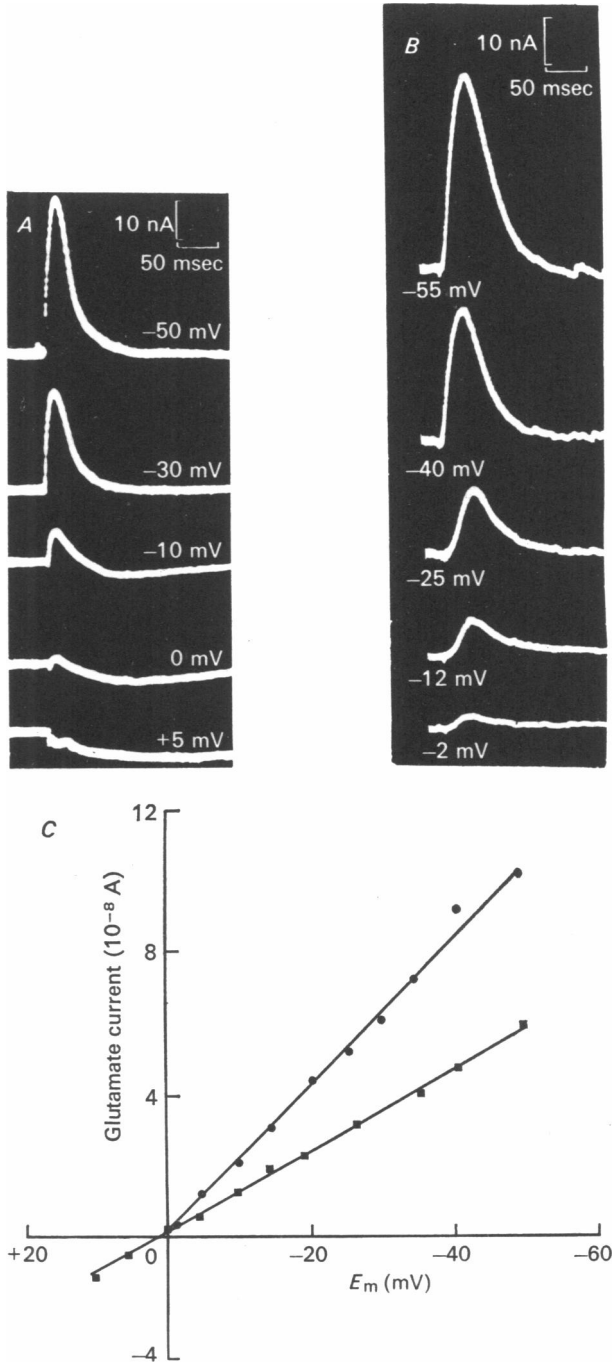

Fig. 5. For legend see facing page.

Effect of ions on the junctional currents

The reversal potentials for glutamate currents and e.p.s.c.s were determined first in standard saline, then after equilibration in salines containing different concentrations of K, Na, Ca, Mg and Cl, and finally after returning the extensor tibiae muscle to standard saline. The response to glutamate iontophoresis became constant 10–15 min after a saline change, but much longer equilibration times (15 min–2 hr) were required for the e.p.s.c. This is not surprising since glutamate currents are normally recorded from synapses located superficially on a muscle fibre, whereas an e.p.s.c. represents the summed activity at a large number of synapses with varying degrees of accessibility.

TABLE 1. Summary of reversal potentials in different salines

Na	K	Cl	Ca	$E_{e.p.s.c.}$ (mV)	$E_{g.c.}$ (mV)
170	10	182	2	+4	+3
170	0	182	2	+2	+3
170	20	192	2	+5	+2
170	40	222	2	+12	—
170	10	0	2	+7	+5
10	10	182	2	−8	—
0	10	182	2	—	−15
0	10	182	0	—	more +ve than −25
170	10	182	50	—	+3

Effect of K

$E_{e.p\ s.c.}$ was determined when external K ($[K]_o$) was altered from the standard 10 to 0, 20 and 40 mM (Table 1).

In 0 mM-$[K]_o$, in which the membrane potential hyperpolarized by about 5 mV, $E_{e.p.s.c.}$ was $+2 \pm 2$ mV (mean ± S.D., four experiments). In 20 mM-$[K]_o$, $E_{e.p.s.c.}$ was $+5 \pm 3$ mV (four experiments). In 40 mM-$[K]_o$, in which the muscle fibre was depolarized by about 30mV, $E_{e.p.s.c.}$ was 12 ± 10 mV (nine experiments). This is an alteration of $E_{e.p.s.c.}$ of 8 mV from the value of normal saline. Neuromuscular transmission was abolished in salines containing more than 40 mM-$[K]_o$.

Fig. 5. *A*, glutamate currents recorded from a single nerve-muscle junction at various membrane potentials in normal saline. *B*, glutamate currents at various membrane potentials in Cl-free saline. Note the absence of the extrajunctional outward current. *C*, relationship between the amplitude of two junctional glutamate currents (■ and ●) and membrane potential in normal saline. Lines through points drawn by eye.

In 0 mM-$[K]_o$, $E_{g.c.}$ was $+3 \pm 3$ mV (four experiments), and in 20 mM-$[K]_o$, $E_{g.c.}$ was $+2 \pm 3$ mV (four experiments). $E_{g.c.}$ could not be determined in higher concentration of $[K]_o$ because of the very high frequency of miniature e.p.s.c.s in this saline.

$E_{g.c.}$ remained at the same value when determined 15 min and 1 h after the perfusion of the test saline.

Effect of Cl

When the chloride in the medium bathing the extensor tibiae muscle was replaced by methylsulphate, a transient depolarization of the muscle fibres occurred (Usherwood & Grundfest, 1965). The final resting potential in Cl free saline was identical to that in standard saline.

$E_{e.p.s.c.}$ was $+5 \pm 3$ mV (four experiments), and $E_{g.c.}$ was $+7 \pm 4$ mV (four experiments) in Cl-free saline.

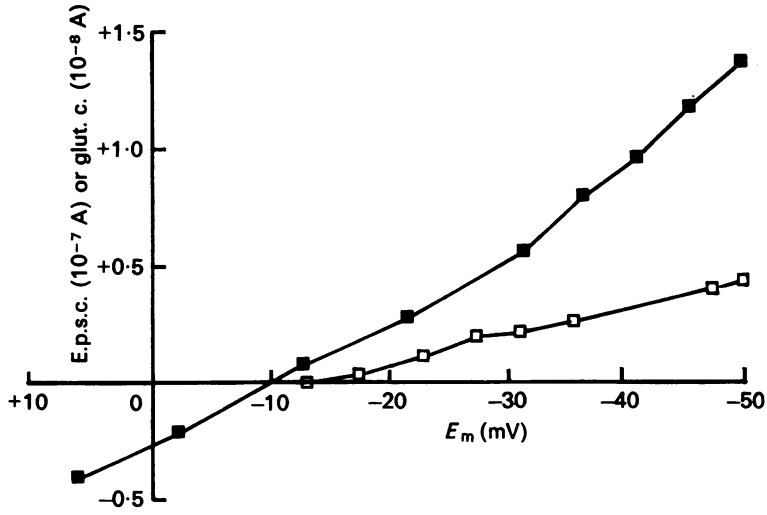

Fig. 6. Relationship between membrane potential and the amplitude of an e.p.s.c. (■) in 10 mM-Na saline, and the amplitude of a glutamate current (□) in Na-free saline.

Effect of Na

Complete replacement of external sodium ($[Na]_o$) by Tris did not change the resting potential of the muscle fibres, although the nerve action potential was abolished. It was not, therefore, possible to examine the e.p.s.c. in Na-free saline. However, in 10 mM external Na a small e.p.s.c. (5–30% of its original amplitude) could be recorded. $E_{e.p.s.c.}$ in 10 mM-Na was -8 ± 5 mV (nine experiments), an alteration of 12 mV from normal saline (Fig. 6).

The glutamate current was also reduced in amplitude when $[Na]_o$ was replaced by Tris (Fig. 7). The reduction in the current amplitude was approximately linearly related to the reduction in $[Na]_o$, although a small current of 5–10% of the original amplitude always remained in Na-free saline containing 2 mM-Ca (Fig. 7).

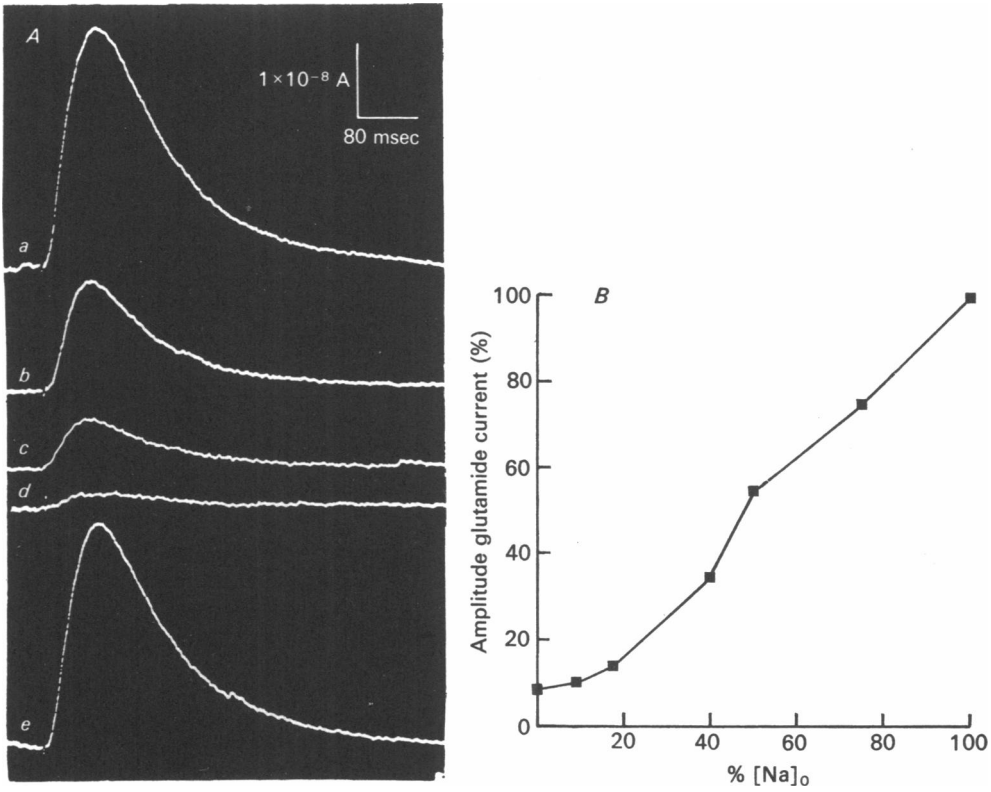

Fig. 7. *A*, progressive reduction in amplitude of a glutamate current caused by perfusion with Na-free saline. Trace *a*, glutamate current in normal saline. Traces *b–d*, glutamate currents 2 min, 5 min and 1 h respectively after perfusion with sodium-free saline. Trace *e*, glutamate current after return to normal saline. Part *B*, shows trace *f* relationship between the amplitude of the glutamate current (expressed as percentage of the amplitude in normal saline) and the concentration of sodium in the saline (expressed as percentage of the Na concentration in normal saline). All measurements made at resting potential.

$E_{g.c.}$ in Na free saline containing 2 mM-Ca was -14 and -16mV in two experiments, an alteration of $E_{g.c.}$ by 18 mV from that in normal saline.

The glutamate receptores showed a very rapid desensitization in Na-free

saline, and a full amplitude glutamate current could only be obtained by a stimulation rate of about 1/min, compared with 1/2–5 sec in normal saline.

Effect of Ca

When the external Ca concentration ($[Ca]_o$) was reduced from its value of 2 mM in standard locust saline to 0·7 mM there was no change in either $E_{e.p.s.c.}$ or $E_{g.c.}$ (Table 1). Any further reduction in $[Ca]_o$ blocked nerve-muscle transmission, but even when $[Ca]_o$ was zero $E_{g.c.}$ remained unchanged. When $[Ca]_o$ was increased above 2 mM the e.p.s.c. increased in amplitude, accompanied by activity in the electrically excitable membrane of the muscle fibre. As a result it was not possible to determine $E_{e.p.s.c.}$. However no change in $E_{g.c.}$ occurred when $[Ca]_o$ was varied between 2 and 50 mM, although when $[Ca]_o$ was increased the amplitude of the glutamate current declined, and in 50 mM-$[Ca]_o$ the glutamate current was only 10% of the amplitude in normal saline.

The small glutamate current seen in Na-free saline was abolished when Ca was completely removed from the saline. Altering the membrane potential by 20 mV in a depolarizing or hyperpolarizing direction in Na-free and Ca-free saline did not result in the appearance of any glutamate current. In a saline containing 10% Na but 0 mM-Ca, only a very small current of 2–3% of the amplitude in normal saline could be recorded. The reversal potential of this current was difficult to determine accurately because of its very small size, but an inward current was still present when the membrane potential was depolarized to −25 mV. $E_{g.c.}$ in 10% Na and zero Ca is, therefore, more positive than −25 mV.

Bath application of L-*glutamate*

When L-glutamate was added to the saline perfusing the locust extensor tibiae muscle an immediate increase in input conductance was observed (Usherwood & Machili, 1966). By removing Cl from the bathing medium and replacing it with the impermanent anion methylsulphate it was possible to eliminate activation of extrajunctional H-receptors (Cull-Candy & Usherwood, 1973). Undoubtedly, when glutamate is applied in Cl-free saline the extrajunctional D-receptors are activated together with the junctional glutamate receptors. However, extrajunctional receptors desensitize much more rapidly than the junctional receptors, and, therefore in the present studies was probably looking mainly at conductance changes originating from glutamate-receptor interaction at junctional as opposed to extrajunctional sites. The muscle fibres were perfused (20 ml. saline/min) with Cl- free saline and clamped at 60 mV. Inward current pulses of 100–500 msec duration were applied to give hyperpolarizations of about 10 mV. In Fig. 8*A* application of L-glutamate at a concentration of

8×10^{-5} M reduced the input resistance of the muscle fibres from 950 to 320 kΩ before desensitization became apparent. Dose–response curves were derived from such experiments for glutamate concentrations ranging between 10^{-5} and 10^{-4} M. An example of such a curve is given in Fig. 8*B*. The limiting slopes of log-log plots varied between 1·7 and 2·1.

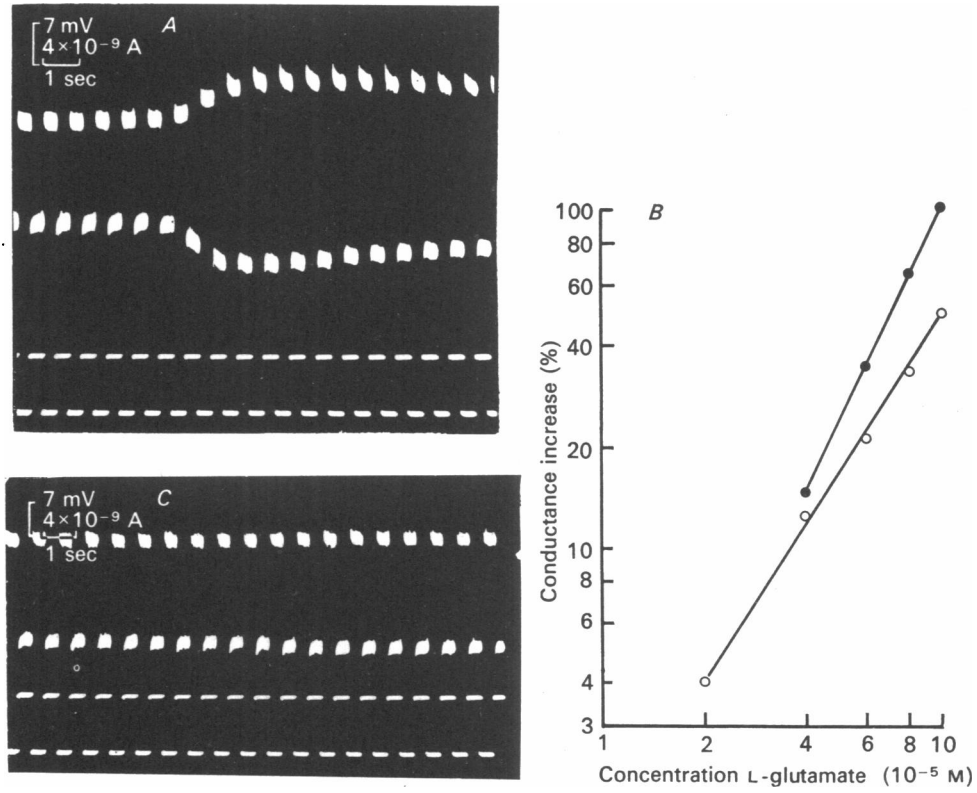

Fig. 8. *A*, effect of bath application of 8×10^{-5} M L-glutamate on a voltage clamped fibre. The top trace shows the current pulses in response to 10 mV hyperpolarizing voltage pulses (lower trace). The perfusion of L-glutamate causes a large increase in the size of the current pulses indicating an increase in conductance. *B*, log-log plot of two dose–response curves for the bath application of L-glutamate. *C*, conductance increase caused by bath application of 2×10^{-3} M L-glutamate to a voltage clamped fibre in Na-, Ca- and Cl-free saline. Note the very small conductance increase despite the very high concentration of L-glutamate.

Bath application of L-glutamate to muscle fibres equilibrated for 3–6 h in Na-free, Ca-free and Cl-free saline caused only a small increase in input conductance. For example, application of 2×10^{-3} M L-glutamate caused

only an 8 % increase in input conductance (Fig. 8*C*) which is about twenty times less than in standard locust saline.

The influence of magnesium

Since for studies on the e.p.s.c. it was necessary to add at least 40 mM-Mg to the bathing medium, the possibility that this ion might influence the properties of the post-synaptic membrane of the locust excitatory nerve-muscle junction could not be ignored. To test for this possibility extensor tibiae muscles were perfused for 1 hr with saline containing 400 mM ethylene glycol before perfusion with standard locust saline. This procedure abolished muscle contraction and also reduced the amplitude of the e.p.s.c. Values of $E_{e.p.s.c.}$ obtained from fibres treated in this manner were similar to those obtained from normal fibres. This suggests that the concentrations of Mg in these studies do not influence $E_{e.p.s.c.}$.

Analysis

The equilibrium potentials for Na and K were calculated as +55 mV and −65 mV respectively, assuming $[Na]_i$ and $[K]_i$ to be 10 and 124 mM respectively (Wood, 1963). E_{Cl} was calculated as −70 mV, assuming $[Cl]_i$ to be 11·0 mM.

The reversal potentials of the e.p.s.c. and glutamate current were found to be close to 0 mV in the present study, suggesting that the synaptic current is the resultant current of a simultaneously occurring inward Na and outward K or Cl current. Other possibilities will be discussed later.

The electrochemical behaviour of synaptic membranes has been most widely analysed by representing each ionic channel as a resistance in series with an e.m.f. (the equilibrium potential for that ion).

Assuming a conductance increase occurs only to Na and K, then from Takeuchi & Takeuchi (1960).

$$\Delta g_{Na}/\Delta g_K = (E_K - E_R)/(E_R - E_{Na}), \quad (1)$$

where Δg_{Na} and Δg_K are in the increases in conductance to Na and K, E_{Na} and E_K are the equilibrium potentials for Na and K, and E_R is the reversal potential of the synaptic current.

At the locust neuromuscular junction, $\Delta g_{Na}/\Delta g_K = 1{\cdot}35$ in normal saline. If $\Delta g_{Na}/\Delta g_K$ stays constant when external K and Na are altered, as at the vertebrate neuromuscular junction (Takeuchi & Takeuchi, 1960), then from eqn. (1), E_R was calculated to vary by 33 and 25 mV for tenfold changes in external Na and K respectively. No such large changes were observed in the present study, which means that if there is only a conductance increase to Na and K, then $\Delta g_{Na}/\Delta g_K$ is changing when external Na and K are altered. For example, $\Delta g_{Na}/\Delta g_K$ increases to 1·9 in 1 mM-$[K]_o$ and decreases to 0·46 in 40 mM-$[K]_o$, assuming no change in $[K]_o$.

The Goldman equation has been used successfully to describe the relative permeability of the resting membrane to Na and K (Hodgkin & Katz, 1949) and has also been used to analyse the synaptic membrane (Ginsborg, 1973; Anwyl & Usherwood, 1975; Ritchie & Fambrough, 1975; Rang, 1975).

Assuming an increase in permeability to only Na and K, and considering the net currrent is zero at the reversal potential of the synaptic current, then from Goldman (1943) and Hodgkin & Katz (1949),

$$E_R = \frac{RT}{F} \log_e \frac{[K]_o + \Delta P_{Na}/\Delta P_K [Na]_o}{[K]_i + \Delta P_{Na}/\Delta P_K [Na]_i}, \quad (2)$$

where ΔP_{Na} and ΔP_K are the increases in permeability to Na and K, R is the gas constant, T the absolute temperature and F the Faraday number.

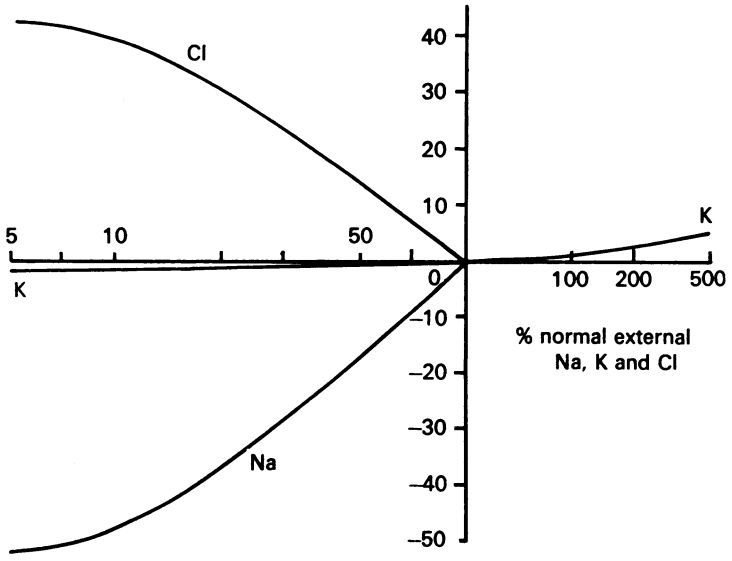

Fig. 9. Changes in the reversal potential of the synaptic current caused by changes in the external concentration of Na, K and Cl. The curves were derived from the Goldman–Hodgkin–Katz equation assuming either P_{Na}/P_K or P_{Na}/P_{Cl} stays constant. The curves for Na and K were calculated assuming the synaptic current is carried exclusively by these ions, and the curve for Cl was calculated assuming the synaptic current is carried by Na and Cl ions.

At the locust neuromuscular junction, $\Delta P_{Na}/\Delta P_K = 0{\cdot}9$ in normal saline. Assuming that $\Delta P_{Na}/\Delta P_K$ remains constant, then from eqn. (2), E_R was calculated to vary by only 12·5 mV when $[K]_o$ is altered from 0 to 100 mM (Fig. 9). E_R is predicted as +2·0 and +8·0 mV in 0 and 40 mM-$[K]_o$ respectively, values which are close to those observed.

When $[Na]_o$ is reduced from 170 to 1 mM, eqn, (2) predicts that E_R will

alter from +3 to −65 mM, providing $[Na]_i$ does not change (Fig. 9). Even if one assumes that $[Na]_i$ is reduced to 1 mM when $[Na]_o$ is 1mM, eqns. (1) and (2) still predict changes of E_R of 30 and 64 mV respectively. Thus both eqns. (1) and (2) predict much larger changes of E_R than observed experimentally when $[Na]_o$ is altered.

It is very unlikely that chloride rather than potassium is carrying the outward synaptic current. Assuming there is a conductance increase to Na and Cl, then eqns. (1) and (2) both predict changes of E_R of about 60 mV when $[Cl]_o$ is reduced to 1mM. However, no change in E_R was observed when $[Cl]_o$ was reduced to 1 mM.

DISCUSSION

The most likely explanation of the results obtained in the present study is that the synaptic current at the locust neuromuscular junction is carried by Na and K, but not Cl, and that an approximately equal permeability increase occurs to Na and K. This conclusion is based on the findings that the reversal potential of the synaptic current is approximately mid-way between the equilibrium potentials for Na and K; that the change of E_R in different external K concentrations is approximately that predicted by the Goldman equation, and also that removing external Na in zero Ca abolishes the synaptic current and causes a substantial shift in E_R. The observation that 5% of the original glutamate current remains when external Na is removed from the normal saline containing 2 mM external Ca indicates that a very small permeability increase to Ca occurs, at least in zero Na.

The most unexpected finding in the present study is that when external Na is removed, E_R changes by only 18 mV, although the Goldman equation predicts a change of over 60 mV. The most plausible hypothesis to explain this finding is that the increase in K permeability caused by L-glutamate and the transmitter is reduced when external Na is lowered, and in zero Na the increase in K permeability is abolished. This explanation is supported by the finding that no iontophoretic glutamate current was observed either at the resting potential or at depolarized or hyperpolarized membrane potentials in Na- and Ca-free saline. Moreover, bath application of a very high concentration of L-glutamate in Na-, Ca- and Cl-free saline caused only a very small conductance increase (8% of that in normal saline). Since $\Delta g_{Na}/\Delta g_K$ is 1·3 in normal saline, a conductance increase of about 45% of that in normal saline would be expected in the Na free saline. The very small conductance increase caused by bath application of glutamate in Na, Ca- and Cl-free saline may be due to residual Na and Ca left in the synaptic clefts.

The hypothesis presented in this paper that the outward current is

dependent upon the inward synaptic current is similar to that proposed for membrane currents in snail neurones (Heyer & Lux, 1976) and skate electroreceptor epithelia (Clusin & Bennett, 1977). Heyer & Lux (1976) found that the outward K current is dependent upon the inward Ca current. Similarly, a late outward K or Cl current in skate electroreceptor epithelia is dependent upon an early Ca current (Clusin & Bennett, 1977). It was suggested that in both of these preparations the inward current initiates the outward current.

The electrochemical behaviour of the synaptic membrane at the locust neuromuscular junction, at which $\Delta g_{Na}/\Delta g_K$ undergoes large changes with alterations of external Na or K, is very different from the frog neuromuscular junction, at which $\Delta g_{Na}/\Delta g_K$ remains constant when external Na and K are altered. However, in recent studies on the ionic permeability of the acetylcholine receptor of cultured rat myotubes, Ritchie & Fambrough (1975) have obtained very similar results to the present study. The activated ACh receptors were permeable to Na and K, with E_R of the ACh potential 3 mV in normal saline. Changes in E_R with external K could be described by the Goldman equation, and altering external Ca did not change E_R. Changes of E_R of 13 mV and 34 mV were found when external Na was reduced to 10 mM with the Na being replaced by Tris and sucrose respectively. The K permeability must be reduced even in sucrose for the Goldman equation predicts a 55 mV change in E_R for a tenfold change in external Na. Moreover, the large shifts in E_R in low Na sucrose saline in the studies of Ritchie & Fambrough (1975) and Takeuchi & Takeuchi (1960) may have been influenced by the changes in the ionic strength of the bathing media, and the Goldman equation applies only to conditions in which the ionic strength is equal on either side of the membrane (Sandblom & Eisenman, 1967). Support for Na and K ions participating in the ACh current of extrajunctional rat muscle receptors was provided by flux studies of Jenkinson & Nicholls (1961). These authors concluded that ACh caused an increase in both Na and K permeability.

There are several other explanations for the results obtained in his study. The lack of a shift of E_R when external K is altered between 0 and 20 mM could be due to the synaptic current being carried only by Na ions. However, this explanation is unlikely because E_{Na} is over 50 mV more positive than E_R, and the observed shift of E_R when external Na is changed is still much smaller than that predicted theoretically. Another possibility is that the synaptic current is carried by K and internal anions. However, this is also unlikely since one would not expect the amplitude or E_R or the synaptic current to be dependent upon external Na.

Table 2 shows the results of previous ionic studies of excitatory receptor membranes. At sites 1–3, changing $[K]_o$ causes a shift in E_R which is close

TABLE 2. Summary of the response of the reversal potential to change of external Na and K at different synapses

Location	Putative transmitter	E_R (mV)	Change of E_R per tenfold change in $[Na]_o$	Change of E_R per tenfold change in $[K]_o$	Reference and original conclusion of ions involved
1. Frog end-plate e.p.p. and ACh	Ach (nicotinic	−15	27–36	15–27	Na and K (Ca). Takeuchi & Takeuchi (1960)
2. Vert. Sympathetic ganglion (fast e.p.s.p.)	ACh (nicotinic)	−15	33	12–18	Na and K. Koketsu (1969)
3. Annelid neuromuscular junction	Ach (nicotinic)	0	10	25	Na and K. Ito, Kuriyama & Tashiro (1969)
4. Cultured rat myotubes	ACh (nicotinic)	−3	33 (sucrose) 8–10 (Tris)		Na and K. Ritchie & Fambrough (1975)
5. Vert. smooth muscle	ACh (muscarinic)	−10	18	5	Bolton (1973)
6. Molluscan C.N.S.	ACh	−12·5	58	0	Na. Levitan & Tauc (1972)
7. Molluscan C.N.S.	ACh	−10	58	0	Na. Blankenship Wachtel & Kandal (1971)
8. Molluscan C.N.S.	Ach	0	32	0	Na. Blankenship Wachtel Kandel (1971)
9. Molluscan C.N.S.	ACh	0	33	0	Na. Chiarandini, Stefani & Gerschenfeld (1967)
10. Insect neuromuscular junction	Glutamate	+4	8–12	0–2	Na and K. Present study
11. Crustacean neuromuscular junction	Glutamate	+12	34	0	Na (Ca). Takeuchi & Onodera (1973)

to that predicted by equation (2) of Takeuchi & Takeuchi. At sites 4–11, changing $[K]_o$ does not cause any appreciable shift in E_R. Several authors whose data are presented in Fig. 1 have concluded that only an increase in conductance to Na occurs at some excitatory synapses. However, as E_R of excitatory synaptic currents lies between +12 and −15 mV, i.e. approximately mid-way between E_{Na} and E_K, and also because the lack of a measurable change in E_R with changes in external K agrees with the predictions of the Goldman equation, it is suggested that it is more likely than an increase in permeability to Na and K occurs. It can be seen from Table 1 that there is a wide variation of change of E_R when $[Na]_o$ is reduced. The Goldman equation predicts a change of 52–57 mV in E_R per tenfold change in $[Na]_o$, and it is postulated that at synapses at which a smaller change in E_R per tenfold change in $[Na]_o$ occurs, the replacement of $[Na]_o$ causes a reduction in the K permeability.

If the ionic permeability of the receptor channels can be described by the Goldman equation, then one would expect the instantaneous Na and K synaptic currents to be non-linear functions of voltage. The calculated Na and K currents at the insect neuromuscular junction have been calculated in Fig. 10 (I_{Na} and I_K) from the equation

$$I_x = -P_K \frac{F^2V}{RT} \left(\frac{[x]_i - [x]_i \, e^{VF/RT}}{1 - e^{VF/RT}} \right), \qquad (3)$$

where x = Na or K; V is the membrane potential; R, T and F have their usual meaning.

It can be seen that the Na and K currents are non-linearly related to voltage, i.e. showing the Goldman rectification. However, when $P_{Na}/P_K = 0{\cdot}9$, as at the insect neuromuscular junction, then the sum of the Na and K current is approximately a linear function of voltage. The non-linear K current should be observed experimentally in Na-free saline. However, this could not be seen at the insect junction because of the reduction in K permeability in Na-free saline. Ginsborg & Kado (1975) have recently observed Goldman rectification in the current–voltage relationship for the increase in K conductance produced by carbachol on inhibitory synapses on *Aplysia* central neurones.

In this study dose–response curves for the action of glutamate on voltage clamped muscle fibres have been carried out using both the bath perfusion and iontophoretic techniques. A fairly large variation occurred using both techniques, with the limiting slope on a log-log plot lying between 1·6 and 2·1 for bath application and 1·6–3·1 for iontophoresis. These results suggest cooperativity in the drug action, perhaps caused by several receptor subunits per channel (Colquhoun, 1973). Similar evidence for cooperativity has been found for the action of cholinergic agonists at the frog

neuromuscular junction (Hartzell, Kuffler & Yoshikama, 1975; Jenkinson & Terrar, 1973), and for the action of GABA on insect muscle (Brookes & Werman, 1973) and on crustacean muscle (Takeuchi & Takeuchi, 1967).

One of the criteria to be satisfied before a substance can be identified as a transmitter is that its action on the post-synaptic cell is in every respect identical to that of the transmitter. The present study has strengthened

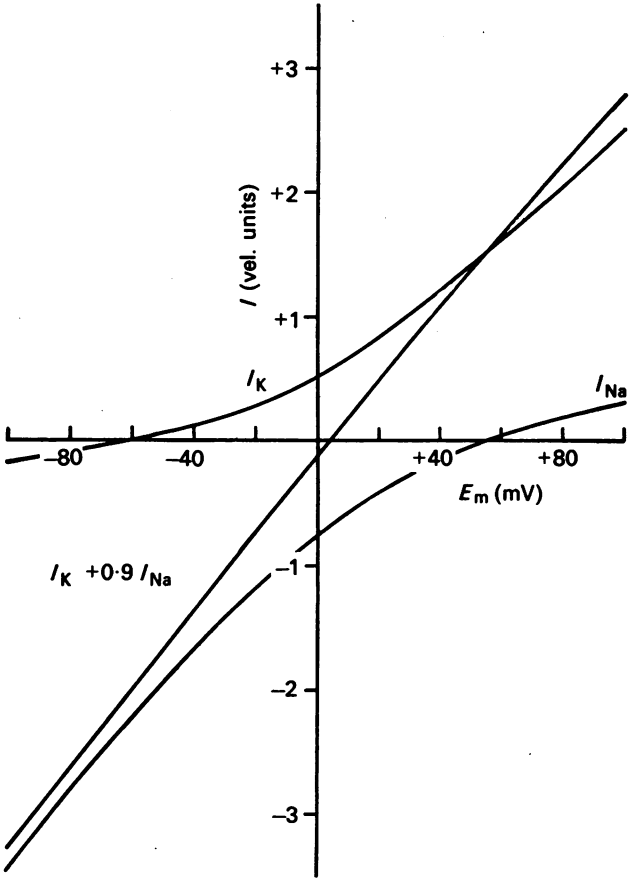

Fig. 10. Current–voltage curves of I_K, I_{Na} and $I_K + 0{\cdot}9\, I_{Na}$ at the locust neuromuscular junction, calculated from the Goldman eqn. (3) assuming $[Na]_i$ and $[K]_i$ to be 10 and 124 mM respectively (Wood, 1963).

the likelihood of L-glutamate being the transmitter substance at the insect neuromuscular junction, for it has been shown that the natural transmitter and L-glutamate cause an identical increase in permeability of the post-synaptic membrane.

I would like to thank Professor P. N. R. Usherwood for invaluable discussion of this work, and Professor T. Narahashi for constructive criticism of this manuscript. This work was supported by an S.R.C. grant to Professor P. N. R. Usherwood.

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