J. exp. Biol. **130**, 405–424 (1987)
Printed in Great Britain © The Company of Biologists Limited 1987

EFFECTS OF TRIMETAPHAN ON LOCUST MUSCLE GLUTAMATE RECEPTORS

BY M. L. J. ASHFORD*, P. BODEN†, R. L. RAMSEY, H. SHINOZAKI‡ AND P. N. R. USHERWOOD

Department of Zoology, University of Nottingham, University Park, Nottingham NG7 2RD

Accepted 3 March 1987

SUMMARY

Effects of trimetaphan have been tested on locust retractor unguis and extensor tibiae nerve–muscle preparations. Trimetaphan reversibly reduced the neurally evoked twitch amplitude of the retractor unguis muscle, dependent upon dose and partly upon stimulus frequency. Contractions evoked by both glutamate (10^{-4} mol l^{-1}) and L-quisqualic acid (10^{-4} mol l^{-1}) were abolished by 1 mmol l^{-1} trimetaphan. Dose–response curves for ionophoresis of L-glutamate were shifted to the right by trimetaphan, with a reduction in peak glutamate potential amplitude. The depression of the ionophoretic glutamate potential was partly dependent upon previous stimulation history, and this action was eliminated by concanavalin A. Trimetaphan had no effect on transmitter release. Within certain limits, trimetaphan reduced the extracellular postsynaptic current (EPSC) amplitude and rise time in a concentration-dependent and voltage-dependent manner. At high concentrations ($>5\times10^{-4}$ mol l^{-1}) its effectiveness was reduced with large hyperpolarizations. The EPSC decay was prolonged and usually biphasic in the presence of trimetaphan, although at some junctional sites a mixture of biphasic and apparently monophasic EPSC decays was seen. It is concluded that trimetaphan exerts more than one effect on locust muscle glutamate receptors. It is likely that it enhances desensitization and also blocks the receptor channel when in the open state.

INTRODUCTION

The anti-hypertensive drug trimetaphan was first reported as a ganglion blocker by Randall, Peterson & Lehmann (1949), a finding subsequently confirmed by Ascher, Large & Rang (1979), Birmingham & Hussain (1980), Taylor (1980) and Rang (1982). The detailed study of Ascher *et al.* (1979) on the mode of action of ganglion blockers on parasympathetic neurones of the rat submandibular gland showed that the action of trimetaphan could be explained purely on the basis of competitive antagonism at these sites.

* Present address: Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD.

† Present address: Parke-Davis Research Unit, New Addenbrookes Hospital Site, Cambridge.

‡ Present address: The Tokyo Metropolitan Institute of Medical Sciences, Bunkyo-Ku, Tokyo, Japan.

Key words: locust muscle, trimetaphan, glutamate receptors.

In addition to this postsynaptic action at cholinergic synapses, trimetaphan has also been shown to block transmission at excitatory nerve–muscle junctions on crayfish leg muscle (Shinozaka & Ishida, 1983) where L-glutamate is the leading transmitter candidate. Evidence was presented that trimetaphan was not a competitive antagonist of L-glutamate and it was suggested that the drug had two major modes of action: first, to decrease the rate of desensitization produced by exogenously applied agonist and second, to shorten the extracellularly recorded synaptic current in a manner consistent with open-channel block.

We decided to investigate in more detail the action of trimetaphan on the well-characterized glutamate receptors found on locust leg muscle where desensitization can be blocked using the plant lectin concanavalin A, allowing us to study the effect of the drug on the glutamate-activated ion channel in isolation, using both voltage-clamp and patch-clamp methods.

MATERIALS AND METHODS

The studies described here were undertaken using two locust nerve–muscle preparations (from either *Schistocerca gregaria* or *Locusta migratoria*); the retractor unguis and extensor tibiae. The advantages of these preparations and their properties are described in detail elsewhere (Usherwood & Machili, 1968; Clark, Gration & Usherwood, 1980). Similar data were obtained from the two locust species. For experiments on retractor unguis muscle (twitch studies) and for ionophoretic studies on extensor tibiae muscle the following saline was used (in $mmol\,l^{-1}$): NaCl, 180; KCl, 10; $CaCl_2$, 2; Na_2HPO_4, 6; NaH_2PO_4, 4; pH 6·8. Some preparations were superfused with concanavalin A (Sigma, Type IV) at a concentration of $3\times10^{-6}\,mol\,l^{-1}$, in locust saline. For focal recording, in extensor tibiae preparations, of extracellular excitatory postsynaptic potentials (EPSPs) and extracellular miniature EPSPs, and for intracellular and single-channel recordings, Hepes ($10\,mmol\,l^{-1}$) was used as the buffer instead of phosphate in order to prevent precipitation with the Mg^{2+} (see below). To reduce the amplitude of the EPSP below the twitch contraction threshold the Ca^{2+} concentration of the saline was reduced to $1·5\,mmol\,l^{-1}$ and $MgCl_2$ was added until an intracellular EPSP of 10–15 mV was obtained (Usherwood, 1972). The final $MgCl_2$ concentration was $25–35\,mmol\,l^{-1}$. Trimetaphan ($10^{-5}–5\times10^{-3}\,mol\,l^{-1}$) was dissolved directly in locust saline. Its structure is shown in Fig. 1.

The influence of trimetaphan on the neurally evoked twitch contractions of the locust isolated retractor unguis nerve–muscle preparation (Usherwood & Machili, 1968) was studied. This preparation contains about 20 muscle fibres divided into two motor units: its small size (1 cm in length and $100\,\mu m$ in diameter) (Rees & Usherwood, 1972) makes it most suitable for pharmacological studies. The apodeme or muscle tendon was attached by a nylon thread to a Grass strain gauge which was connected to a Grass Polygraph recorder. The retractor unguis nerve was stimulated *via* two silver wire electrodes placed in the perfusion chamber at the proximal end of the nerve–muscle preparation.

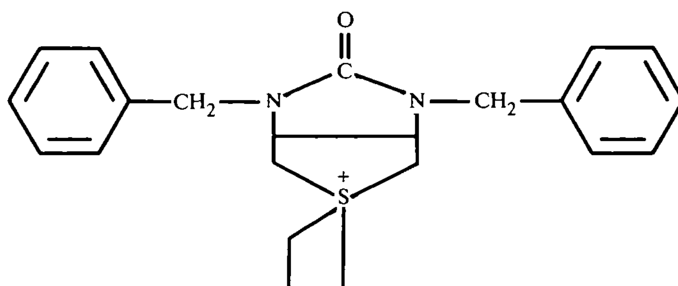

Fig. 1. Structure of trimetaphan.

L-glutamate was applied to single nerve–muscle junctions using high-resistance micropipettes (>100 MΩ) filled with 0·1 mol l^{-1} sodium L-glutamate (pH 8). Small braking currents of +3 to +20 nA were required to prevent background leakage of glutamate from these pipettes. Only glutamate potentials with times to peak of <50 ms were used. Conventional ionophoretic dose–response curves were constructed for data obtained in the presence and absence of trimetaphan using varying doses of L-glutamate, the applications of which were separated by sufficiently long intervals to ensure that the responses did not interact through desensitization and/or potentiation (Walther & Usherwood, 1972; Daoud & Usherwood, 1978; Anis, Clark, Gration & Usherwood, 1981). Trains of pulses of L-glutamate and paired pulses separated by variable intervals were applied to single excitatory junctional sites (Clark, Gration & Usherwood, 1979; Anis *et al.* 1981) in the presence and absence of trimetaphan further to elucidate the effects of this drug on desensitization of the postjunctional membrane.

Focal extracellular EPSPs and extracellular miniature EPSPs were recorded in saline containing Mg^{2+} using saline-filled electrodes of approx. 1 MΩ resistance (Usherwood, 1972). The tips of the electrodes were fire-polished before filling. The extracellular electrodes were connected to a single-ended Bioelectric preamplifier (bandwidth with negative capacitance, 0–30 kHz). Extraneous field potentials were minimized by neurally stimulating only the bundle of muscle fibres under the recording electrode.

EPSCs and miniature EPSCs were recorded intracellularly by a conventional two-microelectrode voltage-clamp (Anwyl & Usherwood, 1974; Anwyl, 1977*a*). Since locust muscle fibres are multiterminally innervated, problems can arise with voltage-clamping if the entire membrane of the muscle fibre is not isopotential (Dekin & Edwards, 1983). However, this is not usually a problem since extensor tibiae muscle fibres are short compared with their length constant (Usherwood, 1963) and the latter can be increased by employing Cl^--'free' saline (impermeant sodium isethionate replacing sodium chloride) (Anderson, Cull-Candy & Miledi, 1978) because locust muscle has a finite chloride permeability (Usherwood & Grundfest, 1965; Lea & Usherwood, 1973). An overall clamp efficiency of 98 % was routinely obtained under these conditions, with the voltage and current electrodes placed within 100 μm of one another in the middle of a muscle fibre (Duce & Scott, 1983). Although we did not routinely check the bandwidth of our clamp, the rise and decay time constants for

the EPSC were similar to those for the extracellular EPSP, which was recorded at a bandwidth of 10 kHz. A non-linear least-squares programme was used to fit as much of the decay phase of the EPSC as possible either by a single exponential function or by the sum of two exponential functions. The time constants of these fits formed the basis of our measurements of decay. No attempts were made to fit more than two exponential functions. The rise time of the EPSC was measured between 20 and 80 % of peak EPSC amplitude, the slope of this component giving a measure of the rate of rise of the EPSC.

In all experiments in which single-channel currents were recorded, the extensor tibiae muscles were pretreated with 3×10^{-6} mol l^{-1} concanavalin A for 30 min to block receptor desensitization (Mathers & Usherwood, 1976, 1978; Patlak, Gration & Usherwood, 1979). The muscle fibres were voltage-clamped to hyperpolarize the membrane and obtain single-channel currents with a good signal-to-noise ratio. Patch electrodes with 1–2 μm diameter tips (after fire polishing) were pressed against the muscle membrane to form mega-ohm seals. The electrodes contained 10^{-4} mol l^{-1} sodium L-glutamate dissolved in locust saline, either alone or with 10^{-4} mol l^{-1} trimetaphan. Recordings were made with a bandwidth of d.c. to 3 kHz and data stored on an analogue FM tape recorder.

All studies were undertaken at room temperature, 20–24 °C. Values quoted in the text and tables represent means ± S.E. or ± S.D. mean. The differences between means were analysed by an unpaired Student's t-test ($P < 0.05$ taken as significant).

RESULTS

Effects of trimetaphan on neurally evoked twitch contractions

When trimetaphan was applied in the superfusing medium to the retractor unguis nerve–muscle preparation, a reversible, dose-dependent reduction in twitch amplitude was observed. For example, after 10–15 min exposure to 10^{-3} mol l^{-1} trimetaphan the steady-state twitch amplitude obtained with a stimulation frequency of 0·16 Hz was reduced by 53·8 ± 5·8 % (mean ± S.D.; $N = 6$). The effect of trimetaphan was also dependent upon stimulus frequency, such that when the stimulus frequency was increased to 2 Hz the amplitude of the twitch was reduced by 68·6 ± 7·6 % ($N = 6$), a value significantly different ($P < 0.05$) from that obtained at 0·16 Hz; the control twitch amplitude at 2 Hz was identical to that recorded at 1 Hz. In preliminary attempts to identify the main site of action of this drug, its effect on the response of the retractor unguis muscle to bath-applied agonist was also investigated. Application of a high concentration (e.g. 10^{-4} mol l^{-1}) of agonist (L-glutamate, L-quisqualate) to a retractor unguis preparation normally evokes a large contraction and reduces the amplitude of the neurally evoked twitch, mainly through receptor desensitization (Usherwood & Machili, 1968); upon removal of agonist the amplitude of the twitch slowly recovers. When either glutamate or quisqualate was applied to a muscle exposed to trimetaphan (10^{-3} mol l^{-1}) it failed to cause a contraction. Trimetaphan was more effective in depressing the contractions evoked by bath-applied agonist than those evoked by nerve stimulation; i.e. even

with $5\times10^{-3}\,mol\,l^{-1}$ trimetaphan the neurally evoked twitch was never completely abolished. Possible explanations for this are that the action of trimetaphan is enhanced in the case of bath-applied agonist by the relatively steady-state concentration of agonist to which the muscle glutamate receptor population is exposed, as opposed to the much briefer, intermittent action of transmitter released by neural stimulation, or simply that the subsynaptic membrane may be less accessible to the drug.

Ionophoretic dose–response relationships

Ionophoresis of L-glutamate onto an excitatory junctional site on a locust muscle fibre evoked a transient depolarization, the amplitude of which was proportional to the amount of L-glutamate ejected from the micropipette (Fig. 2A). To minimize the effects of delayed rectification on the relationship between ionophoretic dose and

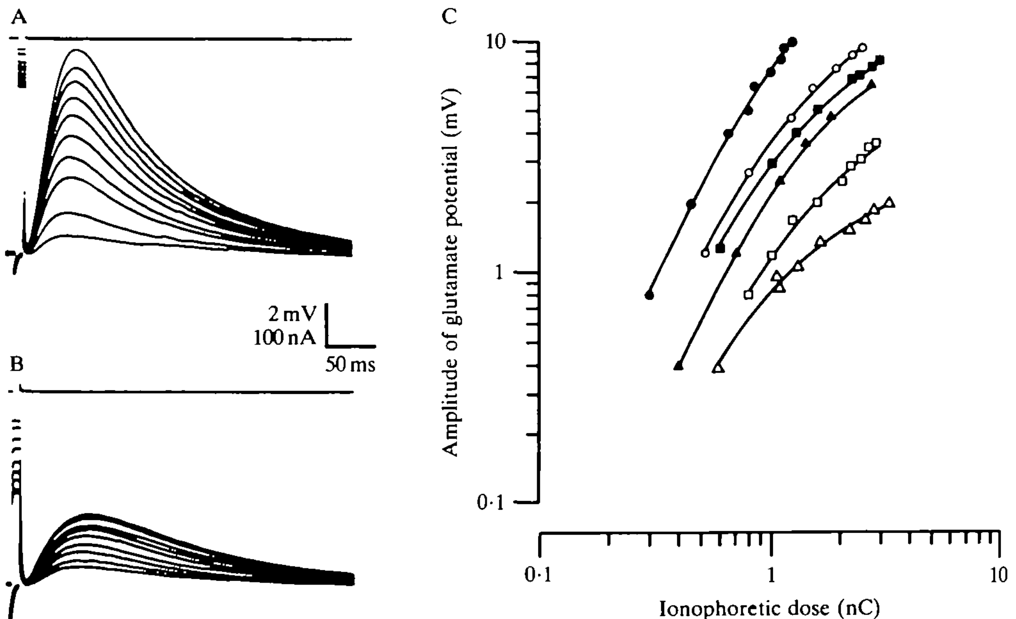

Fig. 2. (A,B) Intracellular recordings (lower traces) of glutamate potentials resulting from ionophoresis of L-glutamate to a superficial excitatory junction site on locust extensor tibiae muscle fibre (resting potential, −62 mV) (A) in standard saline; (B) in $10^{-4}\,mol\,l^{-1}$ trimetaphan (5 min). When the preparation was returned to drug-free saline for 5 min similar responses to those illustrated in A were obtained. The glutamate ejection currents are illustrated in the upper traces. (C) Log–log coulombic dose–response curves constructed from data obtained at a single junctional site. (●) Standard saline; (○) $5\times10^{-5}\,mol\,l^{-1}$ trimetaphan; (■) $7\times10^{-5}\,mol\,l^{-1}$ trimetaphan; (▲) $10^{-4}\,mol\,l^{-1}$ trimetaphan; (□) $3\times10^{-4}\,mol\,l^{-1}$ trimetaphan; (△) $5\times10^{-4}\,mol\,l^{-1}$ trimetaphan. Data were recorded 5 min after exposure to each drug concentration. The preparation was returned to drug-free saline between each application of trimetaphan and the dose–response characteristics were redetermined after 5 min. The effects of the drug were reversible on each occasion. Similar data were obtained from six other superficial junctional sites.

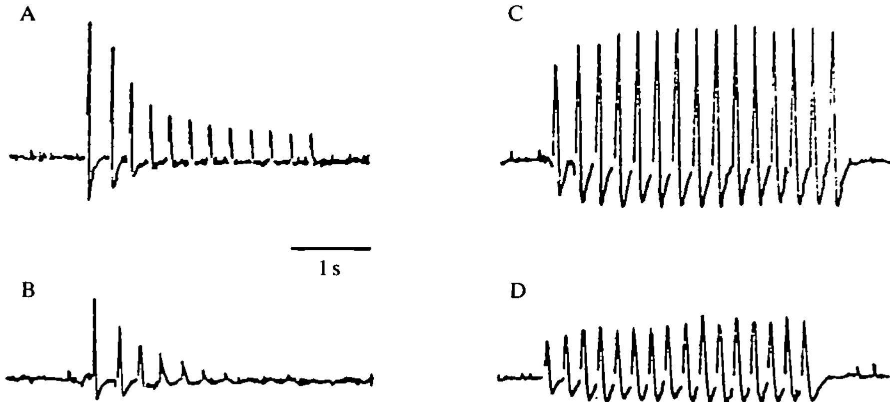

Fig. 3. Depolarizations evoked by trains of L-glutamate pulses applied ionophoretically to a superficial junctional site in saline (A) and after 10 min in saline containing 10^{-4} mol l^{-1} trimetaphan (B). Note that in the presence of trimetaphan the initial response in the train is reduced and the rate of decline in amplitude of the train of glutamate potentials is increased. (C,D) Similar experiments to A and B except that the muscle was pretreated with 3×10^{-6} mol l^{-1} concanavalin A for 30 min. Note that in both C and D there is no evidence for 'desensitization', although in D in the presence of trimetaphan (10^{-4} mol l^{-1}), the amplitude of the glutamate potentials is reduced. The recordings were a.c.-coupled to emphasize the absence of desensitization in C and D. The resting potentials of the muscle fibres were −55 mV (A,B) and −58 mV (C,D). The amplitudes of the initial responses in A and C were 5 mV and 4 mV, respectively. The ionophoretic ejection current was 100 nA; 5 ms. Pulse repetition frequency was 5 s^{-1} in both experiments.

amplitude of the glutamate potential, depolarizations in response to L-glutamate were restricted to the linear region of the voltage–current relationship for locust extensor tibiae muscle (Lea & Usherwood, 1973). In the presence of trimetaphan, which alone had no influence on either muscle membrane potential or input resistance, the amplitude of the glutamate potential was reduced (Fig. 2A,B). Dose–response relationships for L-glutamate ionophoresis were shifted to the right by trimetaphan with a reduction in peak amplitude, the extent of this change increasing as the concentration of trimetaphan was increased (Fig. 2C). The effects of trimetaphan were completely reversible on removal of the drug, provided that there was no movement of the ionophoretic pipette away from the junctional site during an experiment.

Ionophoretic pulse trains

When L-glutamate was applied ionophoretically as brief pulses at frequencies greater than 2 Hz the depolarizations in response to successive pulses declined in amplitude (Fig. 3A), the rate of decline being dependent on the magnitude of the ionophoretic dose and on the pulse repetition frequency (Anis *et al.* 1981). With trimetaphan in the bathing medium, the amplitude of the first pulse in a train was reduced (Fig. 3B) compared with the equivalent response in the absence of the drug (Fig. 3A). The extent of this reduction increased when the concentration of trimetaphan was increased. Trimetaphan also increased the rate at which successive

pulses in a train declined in amplitude, again in a dose-dependent fashion (Fig. 3B). At sites where desensitization onset was monophasic, the decline in amplitude of a train of glutamate potentials in the presence of trimetaphan was also monophasic. Similarly, at sites where desensitization onset in the absence of trimetaphan was biphasic (Anis *et al.* 1981), the depression of the glutamate potentials in a pulse train in the presence of this drug was also biphasic (Fig. 4A). Although these ionophoresis studies were not undertaken using voltage-clamp, it has been established that the onset of desensitization in this system is voltage-independent (Clark, Gration & Usherwood, 1982). At concentrations $>10^{-6}$ mol l^{-1}, concanavalin A blocks desensitization of locust muscle glutamate receptors and it is particularly effective in eliminating desensitization at superficial excitatory junctions on locust muscle fibres (Mathers & Usherwood, 1976, 1978). After treatment with concanavalin A, trains of glutamate potentials both in the presence and in the absence of trimetaphan did not

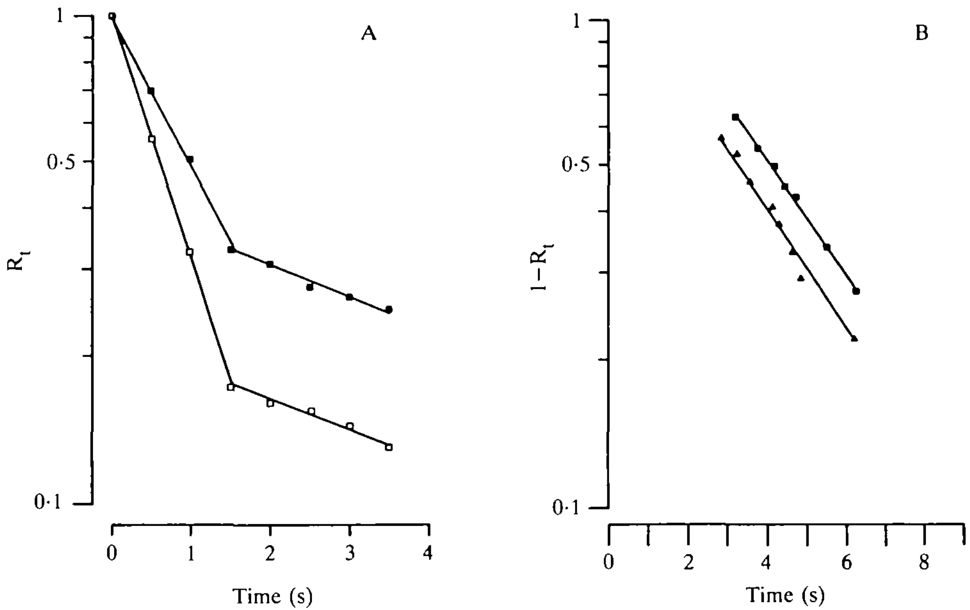

Fig. 4. (A) Effect of trimetaphan on the amplitude of depolarizations evoked at a superficial junction *in response to a train of ionophoretic pulses of* L-glutamate. R_t is the ratio of the amplitude of a response in a train to that of the first response in the train, plotted against time (s). At this site desensitization in the absence of drug (□) comprised two components, an initial fast phase (time constant = 0·19 s) and a second slower phase (time constant = 3 s). In the presence of trimetaphan (■) the decline in amplitude of responses in the pulse train was also biphasic but the initial phase was faster (time constant = 0·16 s). Resting potential, −55 mV. (B) Effect of trimetaphan on recovery from desensitization at another junctional site. Desensitization recovery was measured by delivering a second or 'test' pulse of glutamate from an ionophoretic electrode at different intervals following a first or 'conditioning' pulse delivered from the same electrode. R_t is the ratio of the amplitude of the test pulse to the conditioning pulse. 10^{-4} mol l^{-1} trimetaphan had no effect on the time constant (1 s) for recovery of the 'test' response (i.e. the slope of the best fit line by regression analysis). (■) 10^{-4} mol l^{-1} trimetaphan; (▲) control. Resting potential, −58 mV.

decline in amplitude at frequencies previously found to produce desensitization, although the amplitudes of potentials in the presence of 10^{-4} mol l^{-1} trimetaphan were smaller than those in its absence (Fig. 3C,D). The potentiation of the glutamate responses observed in the concanavalin-A-treated preparations (see Mathers & Usherwood, 1976) was not affected by trimetaphan (Fig. 3C,D).

Double-pulse experiments

Recovery from desensitization at locust glutamate synapses has been studied previously using a variety of techniques (Clark *et al.* 1979; Anis *et al.* 1981). The simplest of these is to examine the effects of variable time intervals between two identical glutamate pulses delivered from a single ionophoretic pipette on the ratio of the amplitude of the second ('test') response to that of the first ('conditioning') response of a pulse pair. Using this technique, Anis *et al.* (1981) showed that recovery from desensitization of locust junctional glutamate receptors is usually an exponential process, the rate constant of which is independent of the level of desensitization. This relationship holds provided that desensitization does not exceed 90 %, when recovery is biphasic (Anis *et al.* 1981). In the present studies where the desensitization in controls was <90 % the rate constant for recovery of 'test' responses in double-pulse experiments was unaffected when trimetaphan (10^{-5}–2×10^{-4} mol l^{-1}) was present in the bathing medium, despite the inhibition caused by this drug (Fig. 4B).

Spontaneous and neurally evoked transmitter release

Intracellular recordings

Trimetaphan at concentrations $>10^{-4}$ mol l^{-1} reduced the amplitude of intracellular miniature EPSPs (Fig. 5) but had no effect on their frequency. It also reduced the amplitude of the intracellular EPSP in a dose-dependent manner. For a given concentration of trimetaphan the intracellular EPSP was less rapidly affected than either the ionophoretic glutamate potential or the extracellular EPSP, and recovery times after removal of drug were longer. The most likely explanation for this is that many of the junctions will be accessible to the bathing solution compared to the superficial junctions only used in extracellular recording.

Extracellular recordings

In five experiments 10^{-3} mol l^{-1} trimetaphan reduced the amplitude of the focally recorded extracellular EPSP by approximately 70 % but the quantal content of the EPSP determined by the 'Method of Failures' (del Castillo & Katz, 1954) remained unaltered (see Table 1). This result, together with the lack of any changes in miniature EPSP frequency in the presence of trimetaphan, suggests that the action of trimetaphan, at least in the concentration range 10^{-5}–10^{-3} mol l^{-1}, is restricted to postsynaptic sites.

In drug-free saline the decay of the extracellular miniature EPSPs recorded at the muscle resting potential of about -60 mV is complex and displays marked variability between events. From the peak of the miniature potential the decay initially follows a

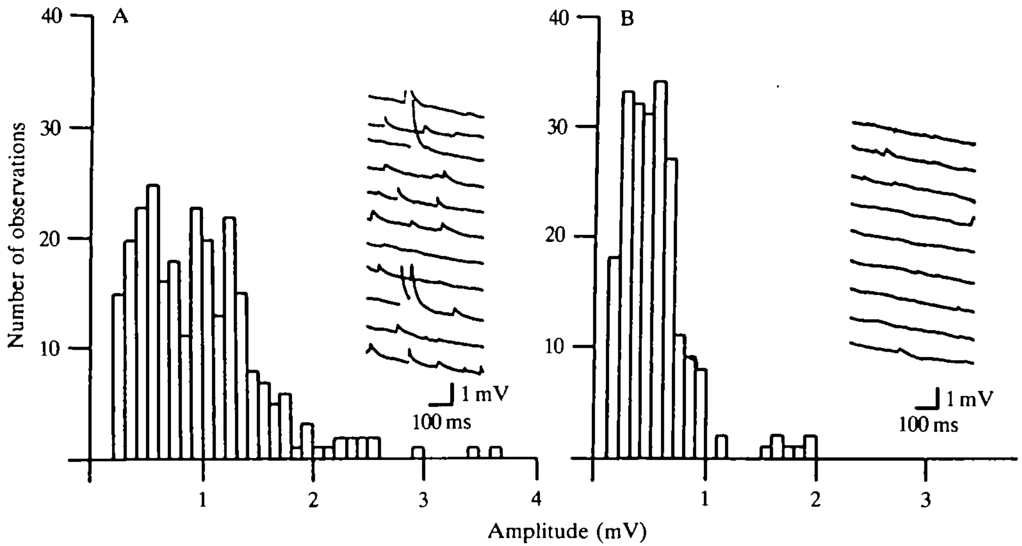

Fig. 5. Histograms showing amplitudes of intracellular miniature EPSPs recorded from a single fibre (resting potential, −60 mV) of a locust extensor tibiae muscle preparation, (A) in saline and (B) in saline containing 10^{-3} mol l^{-1} trimetaphan in the bath solution. Insets show representative data.

non-exponential time course, which is of variable duration (in some cases up to 50 % of the total potential) before becoming exponential (Fig. 6). Occasionally, a late, slow exponential phase or tail is also present. The rise time of the miniature EPSP shows less variation. On addition of trimetaphan (5×10^{-4} mol l^{-1}) to the bathing solution the extracellular miniature EPSP was reduced in amplitude. In addition, many miniature events were characterized by decays comprising two distinct phases (Fig. 6). The miniature EPSP rise time decreased from its value of 0·73 ± 0·02 ms (mean ± S.D., $N = 25$) in the absence of drug to 0·52 ± 0·02 ms in the presence of 5×10^{-4} mol l^{-1} trimetaphan. Because of the complex nature of the miniature EPSP decay observed in controls it was not possible to undertake a full quantitative analysis of the effects of trimetaphan on this component. However, a best fit exponential to the decay phase of control miniature EPSPs gave a mean time constant of 3·02 ± 0·59 ms (S.D., $N = 25$). For the biphasic decays in trimetaphan, the best fit by the sum of two exponentials gave a mean time constant of 1·20 ± 0·25 ms ($N = 25$) for

Table 1. *Quantal analysis of extracellular EPSP by the 'Method of Failures'*

	N_e	N_0	m
Normal saline	133	20	1·89
$+10^{-3}$ mol l^{-1} trimetaphan	114	14	2·10
Normal saline	160	21	2·03

Data obtained from a single site. Similar data were obtained from four other sites.
N_e, total number of applied stimuli.
N_0, number of stimuli which produced no extracellular EPSP.
m, quantal content $\left(= \ln \frac{N_e}{N_0}\right)$.

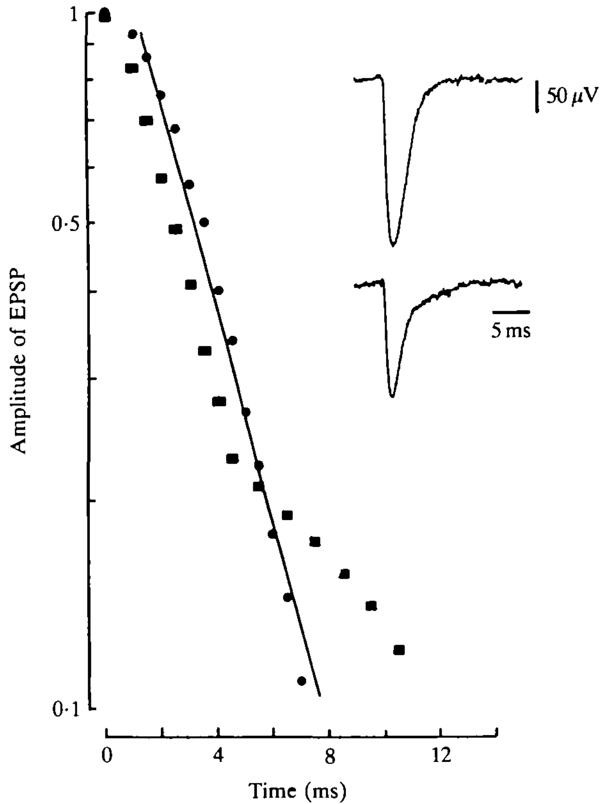

Fig. 6. (A) Semi-logarithmic plot of the decay phase of extracellular miniature EPSPs in saline (●) and in saline containing $5\times10^{-4}\,\mathrm{mol\,l^{-1}}$ trimetaphan (■). The amplitude of the EPSP during its decay is expressed as a percentage of the peak EPSP amplitude. The straight line drawn through the control data was fitted by regression analysis. Note the pre-exponential component of the decay phase of the control current, which is followed by an approximately exponential decay with a time constant of 2·8 ms. The decay phase in the presence of $5\times10^{-4}\,\mathrm{mol\,l^{-1}}$ trimetaphan was biphasic (initial fast phase, time constant 0·37 ms, and late slow phase, time constant 6·7 ms, as fitted by the sum of two exponentials) and the duration of the pre-exponential component was slightly reduced compared with the control. Insets show the currents from which the plotted data were obtained.

an initial fast component and 6·27 ± 0·78 ms ($N = 25$) for a late slow component. (The slow components of some miniature EPSPs had time constants of the order of 17–20 ms: these were not included in the analysis.) These effects of trimetaphan were also observed in muscles which had been pretreated with $3\times10^{-6}\,\mathrm{mol\,l^{-1}}$ concanavalin A. Similar data were obtained for the extracellular EPSP.

Voltage-clamped EPSC

Amplitude

The amplitude of the EPSC varied linearly with membrane potential over the range −40 mV to −140 mV to give an extrapolated reversal potential of

$+2{\cdot}9 \pm 2{\cdot}3\,\mathrm{mV}$ ($N = 5$), a value similar to that obtained from previous studies of EPSCs, glutamate potentials (Anwyl & Usherwood, 1974, 1975) and glutamate-gated channels (e.g. Patlak *et al.* 1979) of locust muscle. Trimetaphan, at all concentrations tested (10^{-5}–$10^{-3}\,\mathrm{mol\,l^{-1}}$), reduced EPSC amplitude ($N = 6$) (Fig. 7A), especially at hyperpolarized potentials, but had no effect on the EPSC

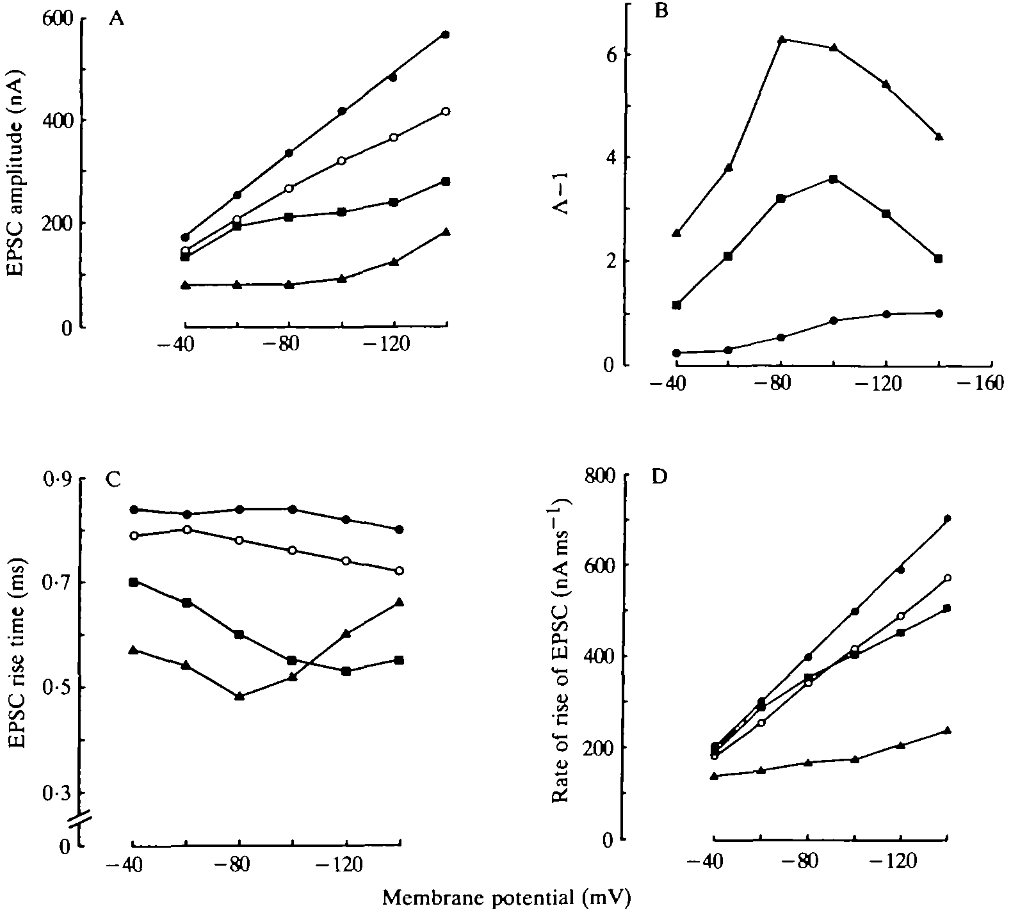

Fig. 7. (A) Dependence of EPSC amplitude on membrane potential in the absence (●) and presence of trimetaphan at concentrations of 10^{-5} (○), 10^{-4} (■) and $5\times10^{-4}\,\mathrm{mol\,l^{-1}}$ (▲). The straight line drawn through the points for the control data was fitted by regression analysis. Each point is the mean from at least 20 EPSCs (S.E. bars are all smaller than the symbols). (B) Depression of EPSC amplitude, expressed as $\Lambda - 1$ (see text for details), as a function of membrane potential for three concentrations of trimetaphan, 10^{-4} (▲), 5×10^{-4} (■) and $10^{-3}\,\mathrm{mol\,l^{-1}}$ (●). Note that there is an optimum membrane potential for high concentrations of trimetaphan, beyond which the effectiveness of the drug is reduced. (C) Dependence of EPSC rise time on membrane potential in the absence (●) and presence of trimetaphan at concentrations of 10^{-5} (○), 10^{-4} (■) and $5\times10^{-4}\,\mathrm{mol\,l^{-1}}$ (▲). (D) Dependence of the rate of rise ($\mathrm{nA\,ms^{-1}}$) of the EPSC on membrane potential in saline (●) and saline containing trimetaphan at 10^{-5} (○), 10^{-4} (■) and $5\times10^{-4}\,\mathrm{mol\,l^{-1}}$ (▲). Each point is the mean for at least 20 EPSCs (S.E. bars are all smaller than the symbols for both C and D).

reversal potential. However, with high concentrations of trimetaphan (10^{-4}–10^{-3} mol l^{-1}) the EPSC amplitude was voltage-independent over parts of the voltage range. For example, with 10^{-3} mol l^{-1} trimetaphan, EPSC amplitude did not change significantly ($P < 0.05$) between −40 mV and −100 mV, although for more negative potentials it was increased. To gain further insight into these phenomena the ratio (Λ) of the control EPSC amplitude and the EPSC amplitude in trimetaphan was determined. A value of 1 for Λ corresponds to no reduction in EPSC amplitude; therefore, $\Lambda - 1$ can be used as an index of the effectiveness of the drug (Ascher *et al.* 1979). Fig. 7B shows that $\Lambda - 1$ varied with trimetaphan concentration and was markedly voltage-dependent. For high concentrations of trimetaphan $>5 \times 10^{-4}$ mol l^{-1}, $\Lambda - 1$ initially increased and then declined as the membrane was progressively hyperpolarized.

Rise time of EPSC

The rise time of the control EPSC in control experiments varied between 0·6 and 1·1 ms, although over the voltage range −40 mV to −100 mV no dependence on membrane potential was observed. However, at membrane potentials greater than −100 mV the rise time was reduced significantly ($P < 0.05$) (Fig. 7C, ●) as compared to values at −40 mV. In the presence of $>10^{-5}$ mol l^{-1} trimetaphan the rise time was significantly reduced compared with controls. However, the effects of trimetaphan concentration and membrane potential on EPSC rise time are complex (Fig. 7C), with the rise time increasing with hyperpolarizations between −80 and −140 mV after exhibiting a decrease with hyperpolarizations between −40 and −80 mV (Fig. 7C, ▲). In this respect the dependence of EPSC rise time on trimetaphan concentration and membrane potential shows a marked resemblance to that of EPSC amplitude.

Rate of rise of EPSC

In the absence of drug the rate of rise of the EPSC increased linearly with membrane potential (Fig. 7D), this relationship being influenced mainly by the concomitant increase in EPSC amplitude. The relationship was less steep when trimetaphan was added to the bathing medium, reflecting both the voltage-dependence of the rise time in the presence of trimetaphan and the influence of this drug on EPSC amplitude.

Decay of EPSC

Normally the EPSC exhibits a decay consisting of two, or sometimes three components (Fig. 8A,B). From the peak of the EPSC there is a non-exponential decay phase (always present in experiments at room temperature) which lasts for the initial 15–20 % of the total current decay. This phase is followed by an exponential component which continues until at least 85–90 % of the decay is complete. Occasionally either a long tail is observed during the last 10–15 % of the decay or this part of the decay is more rapid than the rest.

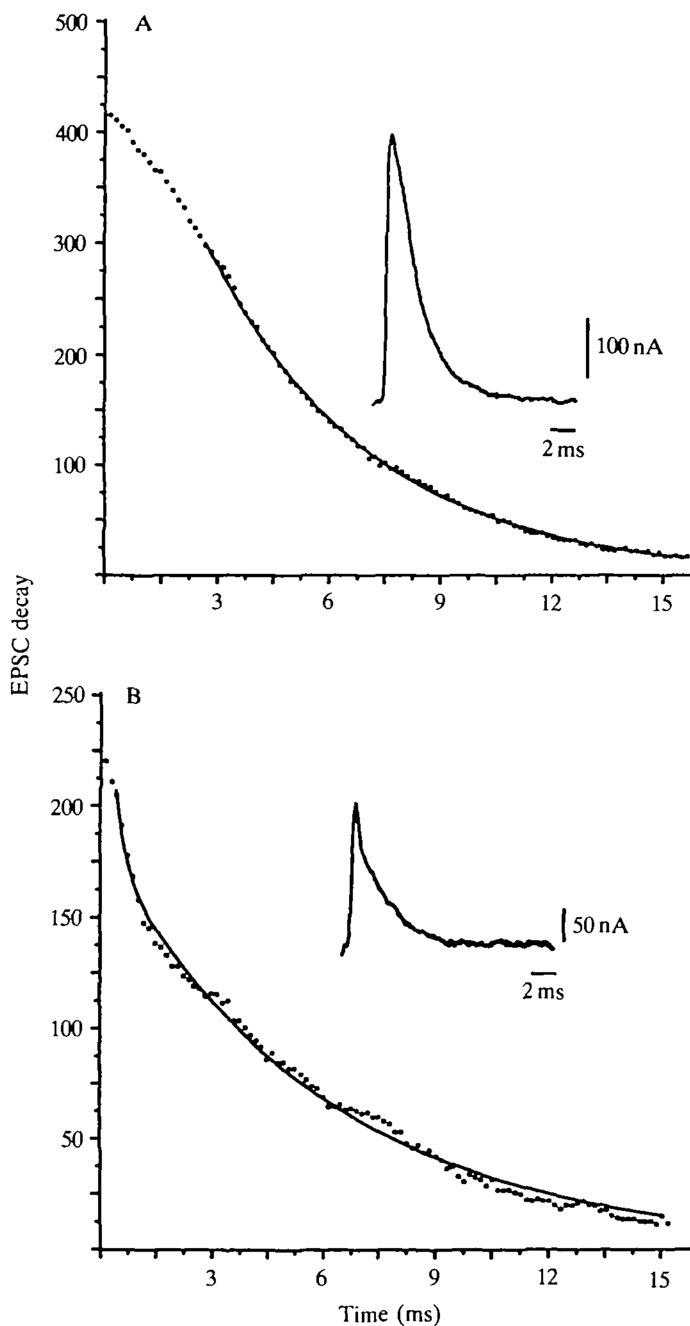

Fig. 8. (A–D) Representative EPSCs in saline (A) and in the presence of 5×10^{-4} mol l^{-1} trimetaphan (B) recorded at a holding potential of −120 mV and plots of their decay phases (A,C, saline; B,D, trimetaphan). The decays of the EPSCs are plotted on linear coordinates in A and C and on semi-log coordinates in B and D. Note biphasic decay of EPSC in trimetaphan. The lines are the best fit exponential(s) to the decays with time constants of 4·4 ms in A and C and 0·26 ms (fast) and 6·0 ms (slow) in B and D.

Values for the decay time constant of the EPSC (τ_{EPSC}), determined by fitting a single exponential to as much of the decay phase as is possible, varied both within and among preparations, the range in six experiments being 2·0–4·5 ms, and exhibited either a weak voltage-dependence or none at all. The decay of the miniature EPSC exhibited similar properties. Anwyl & Usherwood (1975) obtained a τ_{EPSC}

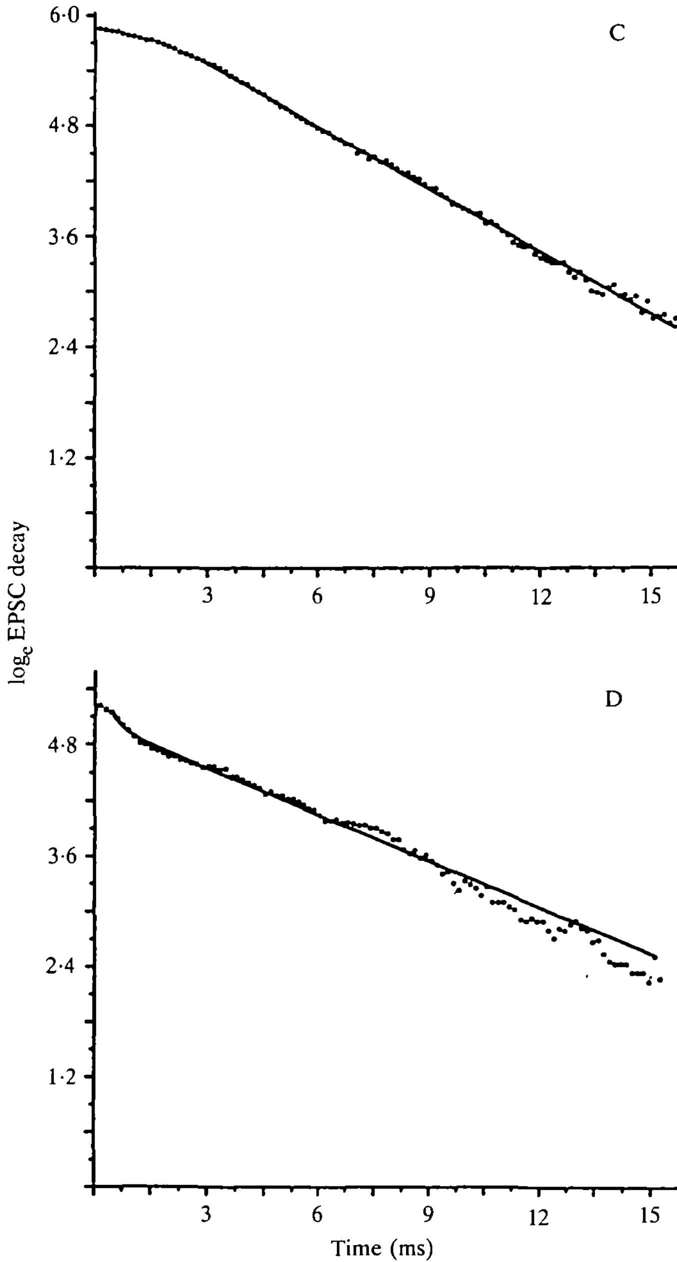

Fig. 8C, D

Table 2. *The effects of trimetaphan on EPSC decay*

Trimetaphan concentration ($mol\,l^{-1}$)	V_m (mV)	Decay time constant (ms)		
		Monophasic	Biphasic 'Fast'	Biphasic 'Slow'
0	−40	4·299 ± 0·075	—	—
	−60	4·358 ± 0·105	—	—
	−80	4·258 ± 0·066	—	—
	−100	4·370 ± 0·077	—	—
	−120	4·415 ± 0·123	—	—
	−140	4·542 ± 0·120	—	—
5×10^{-4}	−40	—	0·65 ± 0·40	5·63 ± 0·58
	−80	—	0·55 ± 0·12	8·77 ± 0·62
	−120	—	0·50 ± 0·10	9·28 ± 0·33
	−140	—	0·32 ± 0·09	9·70 ± 0·58
10^{-3}	−60	—	0·36 ± 0·11	6·90 ± 0·44
	−80	—	0·47 ± 0·14	8·31 ± 1·21
	−100	—	0·46 ± 0·16	8·92 ± 0·53
	−120	—	0·49 ± 0·16	10·44 ± 2·12

V_m, clamped or holding membrane potential of muscle fibre.
All values are means ± S.E. of between 20 and 25 EPSCs for each V_m.

value of 2·8 ms which was also voltage-independent. In contrast, Anderson *et al.* (1978), Cull-Candy & Miledi (1982) and Mathers (1981) have shown that hyperpolarization reduces glutamate receptor channel lifetime and decreases the decay time constant of the miniature EPSC of locust muscle. However, Cull-Candy & Miledi showed that with high divalent cation (Ca^{2+}) concentrations the voltage sensitivity of the decay of the miniature EPSC is reversed. Thus it is possible that the high concentrations of Mg^{2+} used to limit EPSC amplitude in our experiments and in those of Anwyl & Usherwood (1975) may have accounted for the weak or non-existent voltage-dependence of τ_{EPSC}. To test this we measured the voltage-sensitivity of decay of the miniature EPSC recorded from the same muscle fibres in both 0 $mmol\,l^{-1}$ Mg^{2+} and 25 $mmol\,l^{-1}$ Mg^{2+}. In the former case, a weak voltage-sensitivity similar to that observed in previous studies was usually present, but in the latter case the τ_{EPSC} at −140 mV was never significantly different from that recorded at −40 mV.

In general, at concentrations below $10^{-4}\,mol\,l^{-1}$, trimetaphan had little effect on τ_{EPSC}. With higher concentrations (e.g. $5\times10^{-4}\,mol\,l^{-1}$) the EPSC decay became biphasic (Fig. 8C,D) although some apparently monophasic, lengthened decays were also observed. There were no differences in the mean amplitudes of EPSCs with apparently monophasic decays compared to those with biphasic decays. The EPSC decay was also voltage-dependent in the presence of $>5\times10^{-4}\,mol\,l^{-1}$ trimetaphan. Generally the fast phase became faster and the slow phase became slower with membrane hyperpolarization, although with $10^{-3}\,mol\,l^{-1}$ trimetaphan the fast phase did not show any consistent voltage-sensitivity (Table 2). The ratio of the amplitudes of the fast and slow phases was also voltage-dependent, with

hyperpolarization reducing the magnitude of the fast component, and this was particularly evident with the highest concentration (10^{-3} mol l^{-1}) of trimetaphan tested. The apparently monophasic current decays were affected by hyperpolarization in a similar manner to the slow component of the biphasic currents. Hence the predominant effect of high concentrations of trimetaphan ($>10^{-4}$ mol l^{-1}) was an overall lengthening of the EPSC decay which was increased further by hyperpolarization of the muscle fibre. It was also noticeable that the magnitude of the initial non-exponential component of the EPSC decay was reduced; with 5×10^{-4} mol l^{-1} trimetaphan it fell to 5–10% of the total decay at −60 mV and it was almost completely absent at −120 mV.

Single-channel studies

Glutamate, present in the patch electrode at a concentration of 10^{-4} mol l^{-1}, activates extrajunctional D-receptors present in concanavalin-A-treated normally innervated locust muscle fibres resulting in an inward current flow. An example of glutamate-activated single-channel currents recorded from a muscle fibre voltage-clamped to −100 mV is shown in Fig. 9A. At this concentration of glutamate the mean open time of the channel, in the absence of drug, was 0·89 ± 0·06 ms ($N = 12$). When trimetaphan (10^{-4} mol l^{-1}) was present in the patch pipette, in addition to glutamate (10^{-4} mol l^{-1}), the single-channel current amplitude was relatively unchanged but the duration of the individual events was reduced (Fig. 9B). At a holding potential of −100 mV, glutamate-activated ionic channels in the presence of

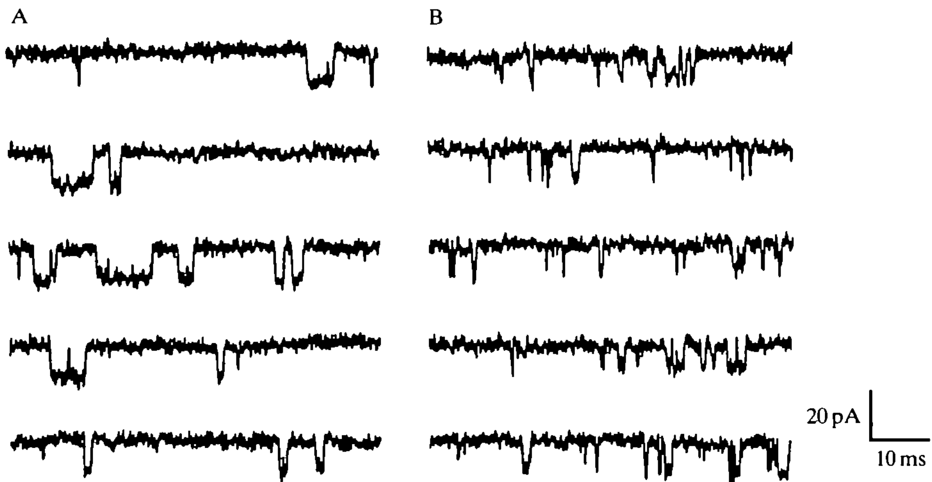

Fig. 9. Single-channel recordings from an extensor tibiae muscle fibre illustrating single-channel currents gated by 10^{-4} mol l^{-1} L-glutamate in the absence (A) and in the presence (B) of trimetaphan (10^{-4} mol l^{-1}). The records in A and B were obtained from separate recording sites on a muscle fibre clamped at −100 mV. Single-channel openings are shown as downward deflections. Note that trimetaphan shortens the duration of the open events, in particular breaking up long openings into a series of bursts of short open and closed periods.

10^{-4} mol l^{-1} trimetaphan had a mean open time of $0{\cdot}62 \pm 0{\cdot}07$ ms ($N = 9$), a significant reduction ($P < 0{\cdot}001$) compared to that in the absence of drugs. This effect of trimetaphan resembles that of many open-channel blocking drugs, for example local anaesthetics and their derivatives (Neher & Steinbach, 1978; Ogden, Siegelbaum & Colquhoun, 1981), on the nicotinic acetylcholine receptor-channel complex.

DISCUSSION

Trimetaphan reduced the amplitude of both the neurally evoked twitch contraction and the ionophoretic junctional glutamate potential of locust muscle and part of this inhibition was related to previous stimulation history. This drug also enhanced the rate at which successive depolarizations elicited by trains of brief pulses of glutamate declined in amplitude. This is in contrast to the results obtained by Shinozaki & Ishida (1983) for the effect of trimetaphan on glutamate receptor-channel complexes in crayfish muscle. These results could be explained if trimetaphan either blocks the open channel gated by the postjunctional glutamate receptor in a manner characterized by rapid block and slow unblock or enhances receptor desensitization (Peper, Bradley & Dreyer, 1982).

It was possible to distinguish between these possibilities in the present study because desensitization of glutamate receptors on locust muscle can be blocked by application of concanavalin A (Mathers & Usherwood, 1976, 1978). Prior treatment of the muscle with this lectin removes the use-dependent decline of the ionophoretic response amplitude both in the absence and in the presence of trimetaphan (Fig. 3C,D). The possibility that concanavalin A acts to prevent this drug from blocking the open glutamate receptor channel can be discounted because the single-channel records clearly show that, even after pretreatment of the muscle with concanavalin A to remove desensitization, trimetaphan can still produce open-channel block (Fig. 9B). In addition, it would be rather coincidental for the unblocking rate of the drug from the open channel to be identical to the rate of recovery of the 'test' glutamate potential in the double ionophoretic pulse studies in the presence and absence of trimetaphan. The single-channel data indicate that the unblocking rate is relatively rapid as trimetaphan breaks up the longer open times into clusters of events, closely spaced in time, a very similar action to that of QX222 on acetylcholine receptor channels in frog muscle (Neher & Steinbach, 1978). Indeed, the only known actions of concanavalin A on locust muscle glutamate receptors are to inhibit desensitization and to suppress the voltage-sensitivity of channel gating (Mathers, 1981). Thus, the simplest explanation of the accelerated depression of trains of glutamate potentials by trimetaphan and its reversal by concanavalin A is that trimetaphan acts to enhance the conversion of the receptor to its desensitized state. Mathers (1981) has suggested that the concanavalin A binding site or sites are discrete from the ion-permeable part of the receptor-channel complex and so it may be that this effect of trimetaphan is the result of interactions with a site or sites other than the receptor channel.

In both the twitch and the ionophoresis studies reported here, part of the inhibition by trimetaphan was independent of previous stimulation history and was unaffected by prior treatment of the muscle with concanavalin A. For example, the first response in a train of pulses was still reduced in the presence of the lectin, although the frequency and dose-dependent decline in successive pulse amplitudes were abolished. This reduction in ionophoretic glutamate potential amplitude by trimetaphan could be explained by some form of channel block. The observations that the reduction in the EPSC amplitude is voltage-dependent and that the ionophoretic dose–response curves have reduced peak glutamate potential amplitudes are not consistent with a purely competitive receptor antagonism. Open-channel block with a fast unblocking rate and/or closed-channel block could account for these effects. Evidence to support an open-channel block mechanism comes from the appearance of biphasic current decays (both in extracellular synaptic potentials and voltage-clamped synaptic currents) which are sensitive to changes in membrane potential, and from the single-channel studies showing that trimetaphan reduces the mean channel open time. These effects are not reversed by pretreatment of the muscle with concanavalin A, which suggests that this drug has at least two distinct actions on the locust muscle glutamate receptor-channel complex: an enhancement of agonist-induced receptor desensitization and a transient block of the open channel.

The effects of trimetaphan on the EPSC parameters require some comment. Open-channel block could account for the biphasic decay and the reduction in the amplitude and rise time of the EPSC by the drug. The voltage-dependence of the two phases of the current decay does appear to fit the sequential model for open-channel block (Adams, 1976, 1977) in that the fast phase becomes faster and the slow phase becomes slower upon hyperpolarization. This model has been largely favoured to account for the action of many charged molecules on vertebrate nicotinic acetylcholine receptor-channel complexes (Peper *et al.* 1982; Lambert, Durant & Henderson, 1983). However, it cannot explain simply many of the anomalies produced by this drug on the EPSC. For example, the appearance of both monophasic and biphasic declining currents at a single recording site, and the recovery of the EPSC amplitude and rise time on large hyperpolarizations. Discrepancies have also been reported at the nicotinic receptor-channel complex. Examples are the action of barbiturates on acetylcholine-induced relaxations in *Aplysia* neurones (Wachtel & Wilson, 1983) and of hexafluorodiethylether on toad miniature EPSCs at low temperature (Gage & Sah, 1982). Gage & Wachtel (1984), studying toad miniature EPSCs, have shown that procaine induces the appearance of both biphasic and monophasic decaying currents at the same recording site. In this respect the locust muscle EPSC, in the presence of trimetaphan, exhibits certain characteristics similar to the action of procaine on the nicotinic system. At present we have no explanation for the reduction in effectiveness of trimetaphan at very negative membrane potentials and high drug concentrations. Further analysis using EPSCs will be unlikely to help due to the complex and variable time course of the synaptic events. Elucidation of these mechanisms must await more detailed studies into the action of this drug at the single-channel level.

The requirement for high concentrations of drug to produce large effects on glutamate-activated channels is consistent with previous studies on glutamate systems. For example, the action of procaine on locust muscle (Anwyl, 1977*b*) and *d*-tubocurarine and the lignocaine derivatives on crayfish muscle (Dekin & Edwards, 1983). One explanation that has been suggested is that there may be structural differences between glutamate-activated and acetylcholine-activated ionic channels (Dekin & Edwards, 1983). In conclusion, the major finding in this study is that one can, for the locust glutamate receptor-channel complex at least, distinguish between open-channel block and enhanced desensitization as a mechanism for use-dependent block by a drug. A useful further study would be to find compounds which act preferentially to enhance desensitization and, in conjunction with the lectins, use these to probe mechanisms of desensitization.

This work was supported by grants to PNRU from the Science and Engineering Research Council and by the award of an MRC Training Fellowship to MLJA. HS was an SERC Senior Visiting Fellow.

REFERENCES

ADAMS, P. R. (1976). Drug blockade of open end-plate channels. *J. Physiol., Lond.* **260**, 531–552.

ADAMS, P. R. (1977). Voltage jump analysis of procaine action at the frog end-plate. *J. Physiol., Lond.* **268**, 291–318.

ANDERSON, C. R., CULL-CANDY, S. G. & MILEDI, R. (1978). Glutamate current noise: post-synaptic channel kinetics investigated under voltage clamp. *J. Physiol., Lond.* **282**, 219–242.

ANIS, N. A., CLARK, R. B., GRATION, K. A. F. & USHERWOOD, P. N. R. (1981). Influence of agonists on desensitization of glutamate receptors on locust muscle. *J. Physiol., Lond.* **312**, 345–364.

ANWYL, R. (1977*a*). Permeability of the post-synaptic membrane of an excitatory glutamate synapse to sodium and potassium. *J. Physiol., Lond.* **273**, 367–388.

ANWYL, R. (1977*b*). The effect of foreign cations, pH and pharmacological agents on the ionic permeability of an excitatory glutamate synapse. *J. Physiol., Lond.* **273**, 389–404.

ANWYL, R. & USHERWOOD, P. N. R. (1974). Voltage clamp studies of glutamate synapse. *Nature, Lond.* **252**, 591–593.

ANWYL, R. & USHERWOOD, P. N. R. (1975). Ionic permeability changes occurring at excitatory receptor membranes of chemical synapses. *Nature, Lond.* **257**, 410–412.

ASCHER, P., LARGE, W. A. & RANG, H. P. (1979). Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *J. Physiol., Lond.* **295**, 139–170.

BIRMINGHAM, A. T. & HUSSAIN, S. Z. (1980). A comparison of the skeletal neuromuscular and autonomic ganglion-blocking potencies of five non-depolarizing relaxants. *Br. J. Pharmac.* **70**, 501–506.

CLARK, R. B., GRATION, K. A. F. & USHERWOOD, P. N. R. (1979). Desensitization of glutamate receptors on innervated and denervated locust muscle fibres. *J. Physiol., Lond.* **290**, 551–568.

CLARK, R. B., GRATION, K. A. F. & USHERWOOD, P. N. R. (1980). Influence of glutamate and aspartate on time course of decay of excitatory synaptic currents at locust neuromuscular junctions. *Brain Res.* **192**, 1205–1216.

CLARK, R. B., GRATION, K. A. F. & USHERWOOD, P. N. R. (1982). Influence of sodium and calcium ions and membrane potential on glutamate receptor desensitization. *Comp. Biochem. Physiol.* **72**C, 1–7.

CULL-CANDY, S. G. & MILEDI, R. (1982). Properties of miniature excitatory junctional currents at the locust nerve–muscle junction. *J. Physiol., Lond.* **326**, 527–551.

DAOUD, M. A. R. & USHERWOOD, P. N. R. (1978). Desensitization and potentiation during glutamate application to locust skeletal muscle. *Comp. Biochem. Physiol.* **59**C, 105–110.

DEKIN, M. S. & EDWARDS, C. (1983). Voltage-dependent drug blockade of L-glutamate activated channels of the crayfish. *J. Physiol., Lond.* **341**, 127–138.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol., Lond.* **124**, 560–573.

DUCE, I. R. & SCOTT, R. H. (1983). GABA sensitivity in the distal bundles of the locust extensor tibiae muscle. *J. Physiol., Lond.* **343**, 31-32P.

GAGE, P. W. & SAH, P. (1982). Postsynaptic effects of some central stimulants at the neuromuscular junction. *Br. J. Pharmac.* **75**, 493–502.

GAGE, P. W. & WACHTEL, R. E. (1984). Some effects of procaine at the toad end-plate are not consistent with a simple channel-blocking model. *J. Physiol., Lond.* **346**, 331–339.

LAMBERT, J. J., DURANT, N. N. & HENDERSON, E. G. (1983). Drug induced modification of ionic conductance at the neuromuscular junction. *A. Rev. Pharmac. Toxicol.* **23**, 505–539.

LEA, T. J. & USHERWOOD, P. N. R. (1973). Effect of ibotenic acid on chloride permeability of insect muscle fibres. *Comp. gen. Pharmac.* **4**, 351–363.

MATHERS, D. A. (1981). The influence of concanavalin A on glutamate induced current fluctuations in locust muscle fibres. *J. Physiol., Lond.* **312**, 1–8.

MATHERS, D. A. & USHERWOOD, P. N. R. (1976). Concanavalin A blocks desensitization of glutamate receptors on insect muscle fibres. *Nature, Lond.* **259**, 409–411.

MATHERS, D. A. & USHERWOOD, P. N. R. (1978). Effects of concanavalin A on junctional and extrajunctional L-glutamate receptors on locust skeletal muscle fibres. *Comp. Biochem. Physiol.* **59C**, 151–155.

NEHER, E. & STEINBACH, J. H. (1978). Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol., Lond.* **277**, 153–176.

OGDEN, D. C., SIEGELBAUM, S. A. & COLQUHOUN, D. (1981). Block of acetylcholine-activated ion channels by uncharged local anaesthetic. *Nature, Lond.* **289**, 596–598.

PATLAK, J. B., GRATION, K. A. F. & USHERWOOD, P. N. R. (1979). Single glutamate-activated channels in locust muscle. *Nature, Lond.* **278**, 643–645.

PEPER, K., BRADLEY, R. J. & DREYER, F. (1982). The acetylcholine receptor at the neuromuscular junction. *Physiol. Rev.* **62**, 1271–1340.

RANDALL, L. O., PETERSON, W. G. & LEHMANN, G. (1949). The ganglionic blocking action of thiophanium derivatives. *J. Pharmac. exp. Ther.* **97**, 48–57.

RANG, H. P. (1982). The action of ganglionic blocking drugs on the synaptic responses of rat submandibular ganglion cells. *Br. J. Pharmac.* **75**, 151–168.

REES, D. & USHERWOOD, P. N. R. (1972). Fine structure of normal and degenerating motor axons and nerve–muscle synapses in the locust *Schistocerca gregaria*. *Comp. Biochem. Physiol.* **43A**, 83–101.

SHINOZAKI, H. & ISHIDA, M. (1983). Trimetaphan as a glutamate inhibitor at the crayfish neuromuscular junction. *Brain Res.* **268**, 296–306.

TAYLOR, P. (1980). Ganglionic stimulating and blocking agents. In *The Pharmacological Basis of Therapeutics*, 6th edn (ed. L. S. Goodman & A. Gilman), pp. 211–219. New York: Macmillan.

USHERWOOD, P. N. R. (1963). Spontaneous miniature potentials from insect muscle fibres. *J. Physiol., Lond.* **169**, 149–160.

USHERWOOD, P. N. R. (1972). Transmitter release from insect excitatory motor nerve terminals. *J. Physiol., Lond.* **227**, 527–551.

USHERWOOD, P. N. R. & GRUNDFEST, H. (1965). Peripheral inhibition in skeletal muscles of insects. *J. Neurophysiol.* **28**, 497–518.

USHERWOOD, P. N. R. & MACHILI, P. (1968). Pharmacological properties of excitatory neuromuscular synapses in the locust. *J. exp. Biol.* **49**, 341–361.

WACHTEL, R. E. & WILSON, W. A. (1983). Barbiturate effects on acetylcholine-activated channels in *Aplysia* neurones. *Molec. Pharmac.* **24**, 449–457.

WALTHER, C. & USHERWOOD, P. N. R. (1972). Characterization of the glutamate receptors at the locust excitatory neuromuscular junction. *Verh. dt. zool. Ges.* **65**, 309–312.