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# THE EFFECT OF A GLUTAMATE UPTAKE INHIBITOR ON AXON–SCHWANN CELL SIGNALLING IN THE SQUID GIANT NERVE FIBRE

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## Summary

The glutamate uptake blocker *p*-chloromercuriphenylsulphonic acid (PCMS) (100 $\mu$mol $l^{-1}$) does not block any of the membrane potential changes induced by the application of L-glutamate to the adaxonal Schwann cells of the giant axon of the tropical squid *Sepioteuthis sepioidea*. This indicates that these potential changes are not due to the activation of an electrogenic glutamate uptake system and supports the idea that they are due to the activation of specific glutamate receptors. The presence of PCMS (100 $\mu$mol $l^{-1}$) reduces the activity of the glutamate uptake system sufficiently for the extracellular level of axonally released glutamate to exceed the threshold for the activation of the NMDA-type glutamate receptors in this preparation.

## Introduction

Glutamate is an important excitatory neurotransmitter both in the vertebrate central nervous system (Monaghan *et al.* 1989) and at the invertebrate neuromuscular junction (Duce, 1988). Recent evidence indicates that non-synaptically released glutamate may also be involved in axon–glial cell signalling, in both invertebrates and vertebrates (see Barres, 1989). The latter role for glutamate was first suggested by Villegas (1978*a*,*b*) to explain the presence of the glutamate-induced release of acetylcholine from the adaxonal Schwann cells of the squid giant axon and the subsequent production of a rapid, long-lasting hyperpolarization of the membrane (see Villegas *et al.* 1988; Evans *et al.* 1991*a*). Recent evidence for the presence of specific glutamate receptors on the squid Schwann cells supports this suggestion (Lieberman *et al.* 1989; Evans *et al.* 1991*a*,*b*). In addition, the finding of specific glutamate receptors on vertebrate glial cells (Marrero *et al.* 1989; Usowicz *et al.* 1989; Wyllie *et al.* 1991) has supported the idea that glutamate may also be involved in axon–glial cell signalling in the vertebrate nervous system.

Glutamate, once released into the nervous system as a neurochemical signal, is thought to be inactivated by a high-affinity uptake mechanism. This may be localised in the

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neurones themselves (see Bennett *et al.* 1974) or preferentially in glial cells both in the invertebrate nervous system (Salpeter and Faeder, 1971; Evans, 1973) and in the vertebrate central nervous system (Schon and Kelly, 1974; Currie and Kelly, 1981). The uptake of glutamate into astrocytes has also been suggested to increase their glycogen content and to reduce glucose utilization (Swanson *et al.* 1990). However, in some glial cell preparations, notably in the vertebrate visual system, the activity of an electrogenic glutamate uptake system has been shown to be responsible for the glutamate-induced depolarization of the glial cells (Brew and Attwell, 1987; Barbour *et al.* 1988; Schwartz and Tachibana, 1990). The presence of glutamate uptake potentials has also been demonstrated in type 1 astrocytes in the first few days in culture, although these cells also develop specific glutamate receptors after a few days in culture (Wyllie *et al.* 1991).

The present investigation was undertaken to determine whether any of the glutamate-induced effects on the membrane potential of the Schwann cell of the giant axon of the tropical squid *Sepioteuthis sepioidiae* were mediated or modulated by the activation of an electrogenic glutamate uptake system. An additional aim was to see whether the presence of an active uptake system for glutamate could explain some of the anomalies observed between the effects of the application of exogenous glutamate and the effects of glutamate released during giant axonal stimulation in this preparation.

## Materials and methods

Giant nerve fibres with a diameter of 300–400 $\mu$m were dissected in sea water from the hindmost stellar nerve of the squid *Sepioteuthis sepioidea*. Giant axons with their surrounding Schwann cell sheaths were then isolated and cleaned of adhering bundles of small nerve fibres by dissection in artificial sea water (see below).

Electrophysiological techniques were as described previously and involved the successive measurements of the electrical potentials of a series of Schwann cells by brief impalements from inside the axon (Villegas, 1972, 1973, 1975; and see Lieberman *et al.* 1989). All experiments were carried out at room temperature (20–22 °C). A blind protocol was used in which the investigator sampling the Schwann cell membrane potentials did not know the identity of the test pulses being applied to the preparation.

Drugs superfused over the surface of the preparation were dissolved in artificial sea water containing 442 mmol $l^{-1}$ NaCl, 10 mmol $l^{-1}$ KCl, 11 mmol $l^{-1}$ $CaCl_2$, 45 mmol $l^{-1}$ $MgCl_2$ and 10 mmol $l^{-1}$ TrisCl buffer (pH 8.0). All the superfused solutions were continuously bubbled with a mixture of 95 % $O_2$ and 5 % $CO_2$. All drugs were obtained from the Sigma Chemical Co.

## Results

The effect of the glutamate uptake blocker *p*-chloromercuriphenylsulphonic acid (PCMS) (Balcar and Johnston, 1972) on the rapid hyperpolarizations of the Schwann cell membrane induced by exposure to short 1 min pulses of L-glutamate has been assessed. Fig. 1 shows that these effects were not blocked in the presence of 100 $\mu$mol $l^{-1}$ PCMS. The threshold for such a rapid L-glutamate-induced hyperpolarization was between

$2\times10^{-10}$ and $5\times10^{-10}$ mol $l^{-1}$ in the absence of the uptake blocker, whilst in its presence the threshold was lowered by almost two orders of magnitude to between $10^{-12}$ and $5\times10^{-12}$ mol $l^{-1}$. The effect of the uptake blocker was reversible and the threshold gradually increased as the blocker was washed out, returning to control levels after about 5 min. In the presence of 100 $\mu$mol $l^{-1}$ PCMS the hyperpolarizing effects of 1 min pulses of $10^{-8}$ mol $l^{-1}$ carbachol were not potentiated (data not shown), indicating that the potentiating effects of PCMS on the responses to L-glutamate are not likely to be on the

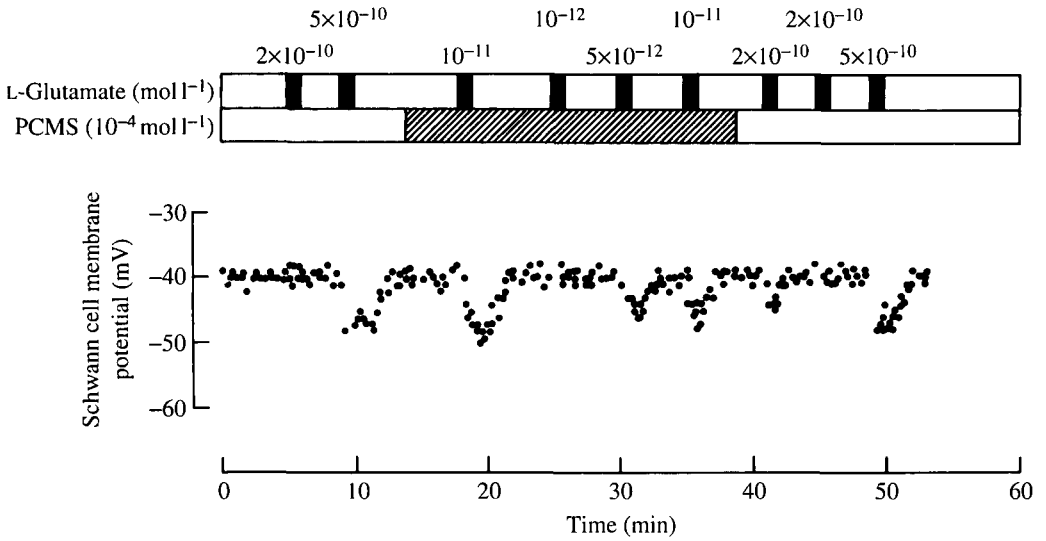


Fig. 1. Actions of 1 min pulses of various concentrations of L-glutamate (filled bars) in the presence and absence of 100 $\mu$mol $l^{-1}$ PCMS (hatched bar) on the Schwann cell membrane potential. Each point represents the potential difference recorded in a different Schwann cell.

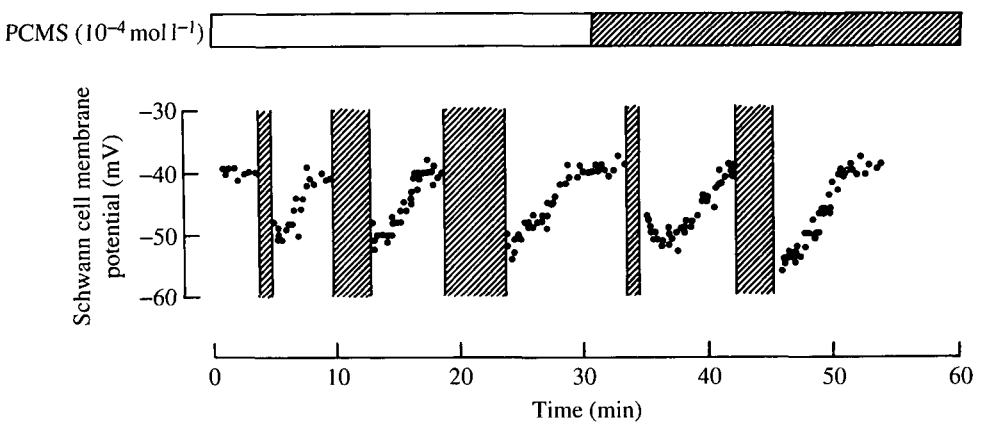


Fig. 2. The effects of stimulating the giant axon at 100 Hz for different periods (vertical hatched bars) in the presence and absence of 100 $\mu$mol $l^{-1}$ PCMS (hatched bar) on the Schwann cell membrane potential. Each point represents the potential difference recorded in a different Schwann cell.

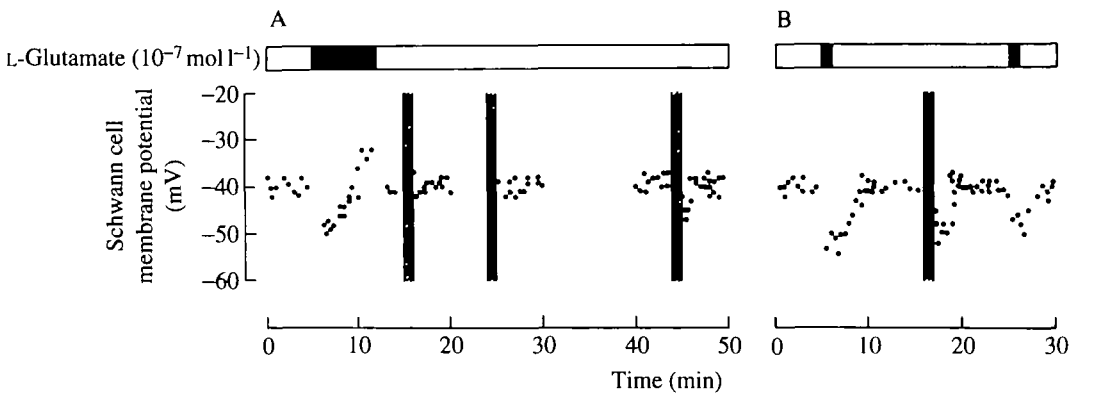


Fig. 3. The effects of stimulating the giant axon at 100 Hz for 1 min (vertical bars) at various times after the exposure of the preparation to $10^{-7}$ mol $l^{-1}$ L-glutamate for 5 min (A) and 1 min (B) (filled bars) on the Schwann cell membrane potential. Each point represents the potential difference recorded in a different Schwann cell.

cholinergic component of the response, but rather are due to the inhibition of glutamate uptake.

The same concentration of the glutamate uptake blocker was also able to potentiate the hyperpolarization of the Schwann cell membrane potential induced by firing the squid giant axon at 100 Hz for varying periods. Fig. 2 shows that increasing the duration of the stimulation period from 1 to 3 and to 5 min increased the duration and the amplitude of the hyperpolarization induced in the Schwann cell membrane. However, in the presence of 100 $\mu$mol $l^{-1}$ PCMS, a 1 min period of stimulation induced a hyperpolarization that was bigger and longer than that induced by a 3 min pulse under control conditions. In addition, in the presence of the uptake blocker, a 3 min stimulation induced a hyperpolarization that was bigger and longer-lasting than that induced by a 5 min pulse under control conditions. This provides further evidence for L-glutamate being the mediator of axon–Schwann cell signalling in the squid giant axon.

Stimulation of the squid giant axon, although capable of mimicking the hyperpolarization of the Schwann cell membrane potential induced by short glutamate pulses, was not able to mimic the biphasic rapid hyperpolarization and following slow depolarization induced by long glutamate pulses (Villegas, 1981). Furthermore, the rapid depolarizations induced by short pulses of glutamate after the initiation of such a slow depolarization were not mimicked by axonal stimulation. Fig. 3A shows such a glutamate-induced biphasic potential where short periods of axonal stimulation at 100 Hz were ineffective at changing the Schwann cell membrane potential immediately after this slow depolarization but 20 min later they could induce a rapid hyperpolarization similar to the effect of control periods of stimulation given immediately after a short glutamate pulse that only produced a hyperpolarization (Fig. 3B).

One explanation for the above effects could be that under normal control conditions not enough glutamate accumulates after axonal stimulation to produce these other effects because of the highly efficient glutamate uptake system in the *Sepioteuthis sepioidea* giant axon preparation. To test this hypothesis, we have stimulated the giant axon for

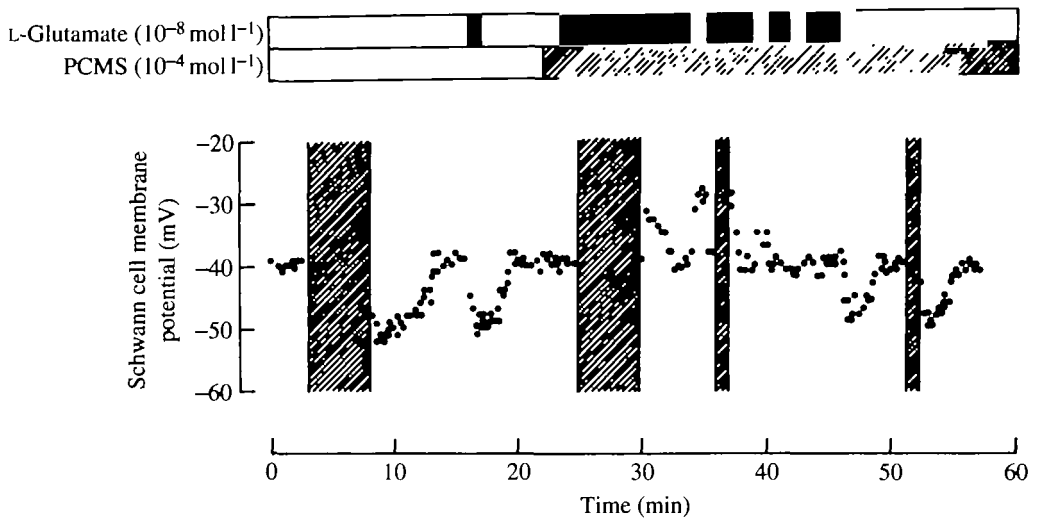


Fig. 4. The effect of stimulating the giant axon for 5 min at 100 Hz in the absence and presence of 100 $\mu$mol $l^{-1}$ PCMS (hatched bar) on the Schwann cell membrane potential. In the presence of the glutamate uptake inhibitor, both 1 min pulses of $10^{-8}$ mol $l^{-1}$ L-glutamate and 1 min periods of giant axon stimulation at 100 Hz produce rapid depolarizing effects after the 5 min period of giant axon stimulation, which itself produces a depolarization. These effects gradually change to hyperpolarizations 15–20 min after the end of the prolonged period of giant axon stimulation. Each point represents the potential difference recorded in a different Schwann cell.

prolonged periods in the presence of the glutamate uptake blocker PCMS at a concentration of 100 $\mu$mol $l^{-1}$. Fig. 4 shows that when the giant axon was stimulated at 100 Hz for 5 min in the presence of the uptake blocker there was a subsequent depolarization of the Schwann cell membrane. This effect was induced by 5 min periods of stimulation but not by 3 min periods of stimulation (cf. Fig. 3) in the presence of the uptake blocker. After the initiation of this depolarization, both a 1 min pulse of $10^{-8}$ mol $l^{-1}$ L-glutamate and a 1 min period of axonal stimlation at 100 Hz induced rapid depolarizations, which slowly reverted to hyperpolarizations, even in the presence of the uptake blocker. Fig. 5 shows that in the presence of the uptake blocker 1 min periods of giant axon stimulation at 100 Hz could also induce transient rapid depolarizations after a slow depolarization had been induced by a long pulse (10 min) of $10^{-8}$ mol $l^{-1}$ L-glutamate, which is similar to the actions of 1 min pulses of $10^{-8}$ mol $l^{-1}$ L-glutamate in the absence of PCMS observed in other preparations (e.g. see Fig. 5A,B in Evans *et al.* 1992). Thus, the inability of axonal stimulation to induce a slow depolarization and subsequent rapid depolarizations in this preparation would appear to be because of the action of an active glutamate uptake system preventing glutamate released upon axonal stimulation from accumulating to sufficiently high concentrations to produce these effects.

We have previously shown that the rapid hyperpolarization and slow depolarization induced by pulses of L-glutamate in the Schwann cell membrane of *Sepioteuthis*

*sepioidea* are likely to be mediated by pharmacologically distinct classes of glutamate receptor (Evans *et al.* 1991*a*,*b*). The rapid hyperpolarization is mediated *via* non-*N*-methyl-D-aspartate (non-NMDA) receptors of the quisqualate/kainate subtype, whilst the slow depolarization is mediated *via* NMDA-subtype receptors. Fig. 6 shows that the slow

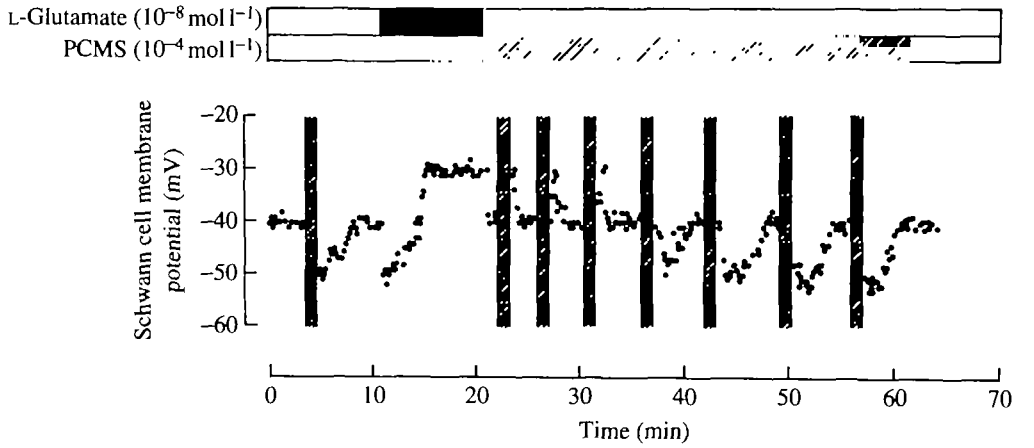


Fig. 5. Time course of change of responsiveness of Schwann cell membrane potential in the presence of 100 $\mu$mol $l^{-1}$ PCMS (hatched bar) to stimulation of the giant axon for 1 min periods at 100 Hz (vertical bars) after the induction of a slow depolarization by exposure to a 10 min pulse of $10^{-8}$ mol $l^{-1}$ L-glutamate (filled bar). Immediately after the slow depolarization, the responses are depolarizing but they revert to hyperpolarizing responses of gradually increasing amplitude 15–20 min after the end of the prolonged glutamate pulse. Each point represents the potential difference recorded in a different Schwann cell.

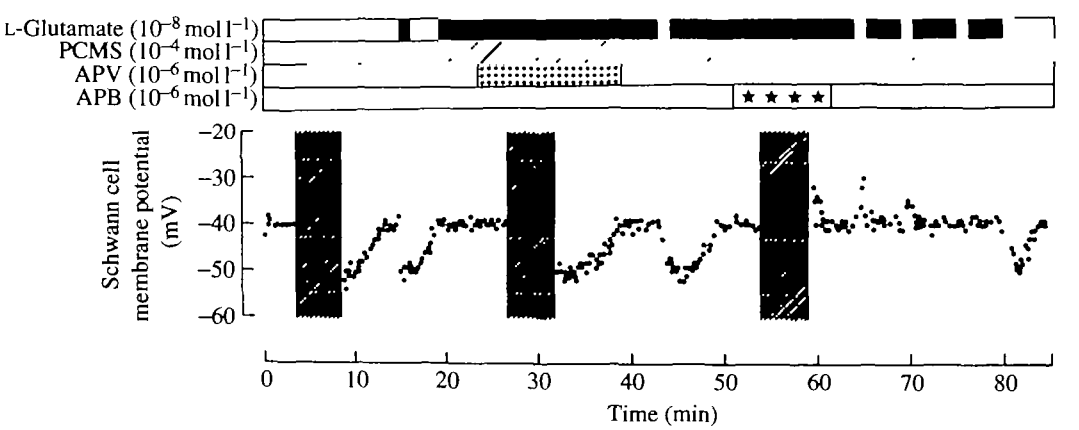


Fig. 6. The depolarizing response of the Schwann cell membrane to a 5 min period of giant axon stimulation at 100 Hz (vertical bars) in the presence of 100 $\mu$mol $l^{-1}$ PCMS is blocked in the presence of $10^{-6}$ mol $l^{-1}$ APV but not in the presence of $10^{-6}$ mol $l^{-1}$ APB, indicating that it is produced by NMDA-type glutamate receptors. After the induction of the depolarization by the prolonged period of giant axon stimulation, the responses to 1 min pulses of L-glutamate (filled bars) are transiently reversed. Each point represents the potential difference recorded in a different Schwann cell.

depolarization induced by stimulating the giant axon for 5 min in the presence of the glutamate uptake blocker was blocked by the specific NMDA blocking agent D,L-2-amino-5-phosphonovaleric acid (APV or AP5) ($10^{-6}$ mol $l^{-1}$) but not by the non-NMDA blocking agent D,L-2-amino-4-phosphonobutyric acid (APB or AP4) ($10^{-6}$ mol $l^{-1}$). After the induction of the depolarization by the 5 min stimulation period in the presence of the uptake blocker and APB, short 1 min pulses of $10^{-8}$ mol $l^{-1}$ L-glutamate again induced transient rapid depolarizations that gradually reverted to hyperpolarizations.

## Discussion

The present study indicates that a high-affinity uptake system for L-glutamate, inhibited by the glutamate uptake blocker PCMS, is present in the giant axon–adaxonal Schwann cell preparation of the tropical squid. This uptake system is not responsible for the generation of any of the changes in membrane potential of the Schwann cell mediated by the release of glutamate from the giant axon, since none of these potentials is blocked by PCMS at concentrations up to 100 $\mu$mol $l^{-1}$. In addition, this conclusion is supported by the pharmacology of the glutamate responses observed in this preparation (see Evans *et al.* 1992). Furthermore, the observations suggest that the glutamate thought to be released from the squid axon during stimulation is unlikely to exit *via* the axonal glutamate carrier. The present study thus supports the idea that the glutamate-induced responses of the squid Schwann cells are mediated by the activation of three distinct classes of specific glutamate receptor (Evans *et al.* 1991*a*,*b*). One type of non-NMDA or metabotropic-type receptor (Evans *et al.* 1992) is responsible for the release of acetylcholine from the Schwann cells and the production of a subsequent nicotinic cholinergic slow hyperpolarization. In addition, two classes of NMDA-type glutamate receptors are present on the Schwann cells, one responsible for the generation of the slow depolarization and a prolonged change in the responsiveness of the Schwann cells, and the other responsible for the production of a fast depolarization during this period of changed responsiveness (Evans *et al.* 1991*a*,*b*; Evans *et al.* 1992). During this time, the glutamate-induced release of acetylcholine is blocked but the Schwann cells are still sensitive to cholinergic agonists such as carbachol (see Villegas, 1984). The presence of a glutamate uptake system has been reported in the axolemma of the squid giant axon (Baker and Carruthers, 1984) and in the Schwann cell plasma membrane (see Villegas, 1984). Schwann cells present in the axoplasm-free sheaths of split giant axon preparations, when exposed to low external concentrations of L-[$^{14}$C]glutamate in the presence of external $Na^+$, are able to accumulate radioactively labelled material in their interior by means of an apparently high-affinity uptake mechanism (R. E. Blanco and J. Villegas, unpublished observations).

The presence of an active uptake system for glutamate also appears to explain some of the discrepancies observed between the effects of exogenous glutamate application and the effects of glutamate released non-synaptically during activation of the giant axon. Stimulation of the giant axon at 100 Hz for periods of up to 5 min in the absence of the glutamate uptake blocker only produces an activation of the non-NMDA-type glutamate receptors, leading to a release of acetylcholine and a subsequent long-lasting nicotinic-

cholinergic-mediated hyperpolarization. However, application of prolonged pulses of L-glutamate at concentrations of $10^{-8}$ mol $l^{-1}$ and above leads to a biphasic effect, consisting of an initial hyperpolarization followed by a slow depolarization, and a change in the responsiveness of the Schwann cell to subsequent glutamate applications as a result of the activation of NMDA-type glutamate receptors (Villegas, 1978*a*,*b*; Evans *et al.* 1991*a*,*b*). The present investigation has shown that in the presence of the glutamate uptake blocker stimulation of the giant axon for periods of 5 min, but not 3 min, can activate this class of NMDA-type glutamate receptor, producing a depolarization of the Schwann cell membrane and a prolonged change in its responsiveness. In addition, in the absence of the uptake blocker, after the activation of the NMDA-type receptors responsible for the slow depolarization and the change in responsiveness of the Schwann cell, short 1-min pulses of L-glutamate cause a depolarization because of the activation of a second class of NMDA-type receptor (Evans *et al.* 1991*a*,*b*; P. D. Evans, V. Reale, R. M. Merzon and J. Villegas, in preparation), whereas stimulation of the giant axon for 1 min periods at 100 Hz has no effect on the Schwann cell membrane for a period of 20–30 min. However, in the presence of the uptake blocker both a 1 min period of giant axon stimulation and the application of a 1 min pulse of L-glutamate induce a rapid depolarization that gradually reverses to become a hyperpolarizing response after a period of 20–30 min. It is likely that the explanation for both these discrepancies lies in the fact that in the absence of the glutamate uptake inhibitor the activity of the glutamate uptake system does not allow the glutamate released non-synaptically during giant axon activity to accumulate to sufficient levels to activate the NMDA-type receptors (threshold between $10^{-9}$ and $10^{-8}$ mol $l^{-1}$, see Evans *et al.* 1992), whilst sufficient glutamate does accumulate to activate the non-NMDA-type glutamate receptors (threshold between $2\times10^{-10}$ and $5\times10^{-10}$ mol $l^{-1}$, see Evans *et al.* 1992). The presence of the glutamate uptake inhibitor PCMS at a concentration of 100 $\mu$mol $l^{-1}$ reduces the activity of the glutamate uptake system sufficiently for the extracellular level of axonally released glutamate to exceed the threshold for the activation of the NMDA-type glutamate receptors in this preparation.

The functional significance of the presence of a very active glutamate uptake system, in a preparation where glutamate is used as a signal molecule from the giant axon to activate a complicated cascade of events in the Schwann cell leading to a change in the potassium permeability of the cells when the giant axon is active, is unclear. The uptake system could be present to protect the neuronal and glial membranes from continuous exposure to the levels of free L-glutamate in the squid haemolymph, so that glutamate-induced changes in the Schwann cell properties could be more closely matched to patterns of axonal stimulation. However, the presence of such an active glutamate uptake system means that the Schwann cells would have a reduced sensitivity to glutamate released from the giant axon during activity, unless some form of spatial separation occurs between the release and uptake sites. Another interesting possibility to consider, however, is that some of the recently discovered aminergic and peptidergic modulators of the squid giant axon–Schwann cell pathway (Reale *et al.* 1986; Evans *et al.* 1986, 1990) may be capable of modulating the sensitivity of the Schwann cell system to released glutamate by changing the effectiveness of the glutamate uptake system in this preparation. Indeed, a

monoaminergic-receptor-mediated modulation of the kinetics of a high-affinity uptake system for glutamate into astrocytes in primary culture has already been reported (Hansson and Rönnback, 1991). In addition, glutamate-induced activation of NMDA receptors in vertebrates stimulates phospholipase $A_2$ to release arachidonic acid, which produces a prolonged inhibition of glutamate uptake into glial (Müller) cells of the salamander retina (Barbour *et al.* 1989). Thus, the possibility of modulation of the activity of the glutamate uptake system requires further investigation in the Schwann cells of the squid giant axon.

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