

A COMPARISON OF THE RELEASE OF A VASOACTIVE-INTESTINAL-PEPTIDE-LIKE PEPTIDE AND ACETYLCHOLINE IN THE GIANT AXON–SCHWANN CELL PREPARATION OF THE TROPICAL SQUID *SEPIOTEUTHIS SEPIOIDEA*

PETER D. EVANS^{1,*}, VINCENZINA REALE¹, ROSA MARIA MERZON² AND JORGE VILLEGAS²

¹The Babraham Institute Laboratory of Molecular Signalling, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK and ²Centro de Neurociencias, Instituto Internacional de Estudios Avanzados (IDEA), Apartado 17606, Parque Central, Caracas 1015-A, Venezuela

*e-mail: pde10@mole.bio.cam.ac.uk

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Summary

A vasoactive intestinal peptide (VIP)-like peptide is released by axonal stimulation in the giant axon–Schwann cell preparation from the tropical squid *Sepioteuthis sepioidea*. It is also released by direct application of L-glutamate, the giant axon–Schwann cell signalling molecule in this preparation. The release of the peptide parallels the release of acetylcholine from the Schwann cells themselves in this preparation in a number of different ways. The release of both acetylcholine and the VIP-like peptide have the same threshold (between 2×10^{-10} and 5×10^{-10} mol l⁻¹) for L-glutamate application and the same recovery time

after inhibition of release by exposure of the preparation to a prolonged pulse of L-glutamate. A prolonged L-glutamate pulse of 10^{-8} mol l⁻¹ releases both substances for as long as the pulse is applied to the preparation, whereas a prolonged pulse of 10^{-9} mol l⁻¹ L-glutamate releases acetylcholine in the same way but releases the VIP-like peptide only transiently. The VIP-like peptide is likely to be co-released with acetylcholine from the Schwann cells.

Key words: glia, peripheral nerve, acetylcholine, neuropeptide, squid, *Sepioteuthis sepioidea*.

Introduction

The co-release of multiple neuroactive substances from a single neurone is now accepted as part of the complex signalling pathway between cells in the nervous system (Hökfelt et al., 1987; Bartfai et al., 1988; Kupfermann, 1991; Church et al., 1993). In many neuronal preparations that co-release a peptidergic and a non-peptidergic signal, the latter is used for fast, and the former for slow, chemical signals (Iversen, 1984). Thus, for instance, a co-release of acetylcholine and vasoactive intestinal peptide (VIP) has been demonstrated upon stimulation of the parasympathetic nerve innervation to the cat submandibular salivary gland (Lundberg, 1981; Lundberg et al., 1982). Current evidence suggests that a range of neuroactive substances may also be synthesised and released from glial cells (see Martin, 1992). In the present paper, we examine the possibility that a peptidergic and a non-peptidergic signal may also be co-released from a glial cell preparation.

Studies on the neuronal–glial cell interactions in the giant nerve fibre from the stellate nerve of the tropical squid *Sepioteuthis sepioidea* have demonstrated a complex form of signalling between the giant axon and its surrounding adaxonal glial (or Schwann) cells (Villegas, 1981, 1984; Villegas et al., 1988; Evans et al., 1991b, 1995; see also Lieberman et al., 1994; Messenger, 1996). In this preparation, glutamate has been suggested to be released non-synaptically upon

stimulation of the giant axon (Villegas, 1978a,b) and acts on specific glutamate receptors on the Schwann cells (Lieberman et al., 1989; Evans et al., 1991a). The activation of a set of N-methyl-D-aspartate (NMDA) subclass of glutamate receptors on the Schwann cells leads to plastic changes in the response properties of the Schwann cells for a period of 10–15 min (Evans et al., 1991a, 1992). Activation of a separate subclass of metabotropic glutamate receptors (Evans et al., 1992) leads to a release of acetylcholine from the Schwann cells themselves (Villegas and Jenden, 1979), which feeds back to activate nicotinic cholinergic receptors on the Schwann cells (Villegas, 1975). The activation of these receptors increases the membrane permeability of the cells to K⁺ via a cyclic-AMP-dependent pathway (Evans et al., 1985). Many of the above components of the signalling system can be modulated by the biogenic amine octopamine (Reale et al., 1986) and by a range of neuropeptides (Evans et al., 1986). The latter include a VIP-like peptide which appears to be released in this preparation at the same time as acetylcholine during axonal stimulation (Evans et al., 1986; Evans and Villegas, 1988). The VIP-like peptide can also be released in this preparation by the application of L-glutamate, which bypasses the axonal activation stage in the signalling pathway between the axon and the Schwann cells. This was demonstrated in experiments where L-glutamate was shown to be capable of causing a

Schwann cell hyperpolarization which was blockable by a specific VIP-blocking agent after irreversible blockage of the nicotinic cholinergic receptors on the Schwann cells with α -bungarotoxin and potentiation of the effects of the endogenously released neuropeptide by the biogenic amine octopamine (Evans et al., 1995).

The present paper compares the properties of the release of the VIP-like peptide and of acetylcholine in the squid giant axon-Schwann cell preparation under a number of different conditions.

Materials and methods

Giant nerve fibres with a diameter of 300–400 μm 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).

The electrophysiological techniques used in the present study are based on the successive measurements of electrical potentials from a series of individual Schwann cells by brief impalements from inside the axon (Villegas, 1972, 1973, 1975). They have also been described in detail in subsequent publications (see Brunder and Lieberman, 1988; Lieberman et al., 1989; Lieberman and Sanzenbacher, 1992; Evans et al., 1990, 1991a, 1992). In addition, work by Brown et al. (1991) and by Brown and Abbot (1992) has validated the 'transient sampling' technique used here by showing that the same membrane potential profiles can be obtained using long-term microelectrode impalements of squid Schwann cells in split axon preparations. All experiments were carried out at room temperature (20–22 °C). In experiments where the degree of hyperpolarization of the Schwann cell membrane by a given drug has been quantified, it was calculated as the difference between the mean value of the control resting potential in the 2 min period immediately before drug application (averaged over 5–10 penetrations of individual Schwann cells) and the mean value of the membrane potential in the 2 min period immediately after the end of the drug pulse (averaged over 5–10 penetrations of individual cells). The results are given as the mean (\pm S.E.M.) hyperpolarization (in mV) induced by a drug using results obtained from at least four axons. A blind protocol was used in which the investigator sampling the Schwann cell membrane potential did not know the identity of the test pulses being applied to the preparation. All the experiments shown were repeated at least three times in the format shown.

Drugs were superfused over the surface of the preparation and were dissolved in artificial sea water containing 442 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 11 mmol l^{-1} CaCl₂, 45 mmol l^{-1} MgCl₂ and 10 mmol l^{-1} Tris-Cl buffer (pH 8.0). All the superfused solutions were continuously bubbled with a mixture of 96% O₂ and 5% CO₂. All drugs were obtained from the Sigma Chemical Co., except for (pCl-D-Phe⁶, Leu¹⁷)VIP (porcine), which was obtained from Bachem, UK Ltd, and

purified α -bungarotoxin, which was kindly supplied by Dr Michael Raftery of the Department of Chemistry, California Institute of Technology, USA.

Results

Glutamate-induced release of acetylcholine and a VIP-like peptide

To investigate the relationship between the release of the VIP-like peptide and acetylcholine in the squid giant axon preparation, we performed experiments to bypass the electrical activation of the giant axon by directly activating the Schwann cells by the application of L-glutamate, the presumed axon-Schwann cell signalling factor. Fig. 1A indicates that a threshold for an induction of a hyperpolarizing response in the Schwann cells occurs between 10^{-10} and 10^{-9} mol l^{-1} for the application of 1 min pulses of L-glutamate. Increasing concentrations of L-glutamate produce dose-dependent increases in the size of the Schwann cell hyperpolarization, with a maximum effect occurring at concentrations of 10^{-8} mol l^{-1} and above (Fig. 1A,B). However, as noted previously (Evans and Villegas, 1988; Evans et al., 1995), this hyperpolarizing response is actually a composite response. It consists of a true cholinergic response that has been potentiated by the simultaneous release of small amounts of an endogenous VIP-like peptide, which by itself is not released at a high enough concentration to elicit direct changes in Schwann cell membrane potential. The true size of the cholinergic component of the Schwann cell response to L-glutamate can be estimated in experiments where the responses to 1 min pulses of L-glutamate are compared in the presence and absence of the VIP receptor blocking agent (pCl-D-Phe⁶, Leu¹⁷)VIP (10^{-9} mol l^{-1} ; Fig. 1A). This blocking agent preferentially blocks VIP-mediated hyperpolarizing responses in the Schwann cells of the squid giant axon and has no effect on the hyperpolarizing responses generated by the application of octopamine, carbachol or substance P to these cells (Evans and Villegas, 1988). The true cholinergic component of the response has a smaller amplitude and a shorter time course compared with the full response, especially at low concentrations of L-glutamate, but the threshold for the response still occurs at L-glutamate concentrations between 10^{-9} and 10^{-10} mol l^{-1} .

Fig. 1B compares the concentration-dependence of the maximum amplitude of the true cholinergic response with that of the full hyperpolarization response induced by application of 1 min pulses of L-glutamate at different concentrations to the Schwann cells. A significant reduction (Student's *t*-test) in amplitude of the glutamate-induced hyperpolarization was obtained in the presence of the VIP-blocking agent (10^{-9} mol l^{-1}) at glutamate concentrations of 5×10^{-10} mol l^{-1} ($P < 0.001$, $N = 6$) and 10^{-9} mol l^{-1} ($P < 0.05$, $N = 6$), demonstrating the co-release of the VIP-like peptide and its potentiating effect on the true cholinergic responses in this concentration range.

The above experiments indicate that the threshold concentrations for the release of both acetylcholine and the

endogenous VIP-like peptide occur between 2×10^{-10} and $5 \times 10^{-10} \text{ mol l}^{-1}$ L-glutamate (Fig. 1B). However, since the release of the VIP-like peptide could be detected only by blocking its potentiating actions on the true cholinergic

hyperpolarizing responses in these experiments, they do not prove that the VIP-like peptide is not released at lower glutamate concentrations. To address this question directly, we have examined the dose-dependence of the size of the

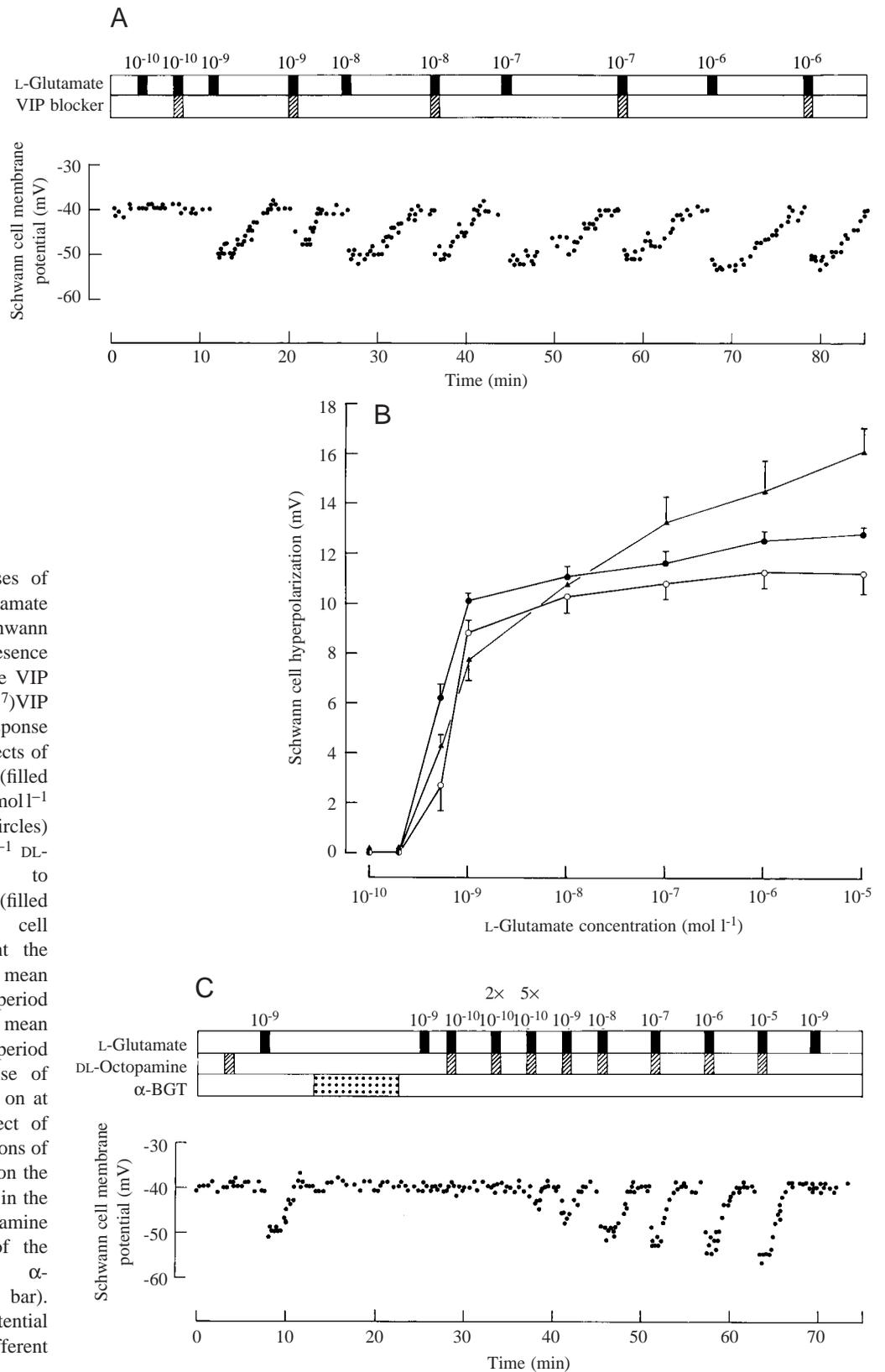


Fig. 1. (A) Effect of 1 min pulses of various concentrations of L-glutamate (mol l^{-1}) (filled bars) on the Schwann cell membrane potential in the presence and absence of $10^{-9} \text{ mol l}^{-1}$ of the VIP blocking agent (pCl-D-Phe⁶, Leu¹⁷)VIP (hatched bars). (B) Dose-response curves for the hyperpolarizing effects of 1 min pulses of L-glutamate alone (filled circles), in the presence of $10^{-9} \text{ mol l}^{-1}$ of the VIP blocking agent (open circles) and in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine after exposure to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (filled triangles) on the Schwann cell membrane. The values represent the difference (\pm S.E.M.) between the mean membrane potential in the 2 min period before agonist application and the mean membrane potential in the 2 min period after the end of the 1 min pulse of agonist. Each solution was tested on at least four nerve fibres. (C) Effect of 1 min pulses of various concentrations of L-glutamate (mol l^{-1}) (filled bars) on the Schwann cell membrane potential in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bars) after exposure of the preparation to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (α -BGT) (stippled bar). Each point represents the potential difference recorded in a different Schwann cell.

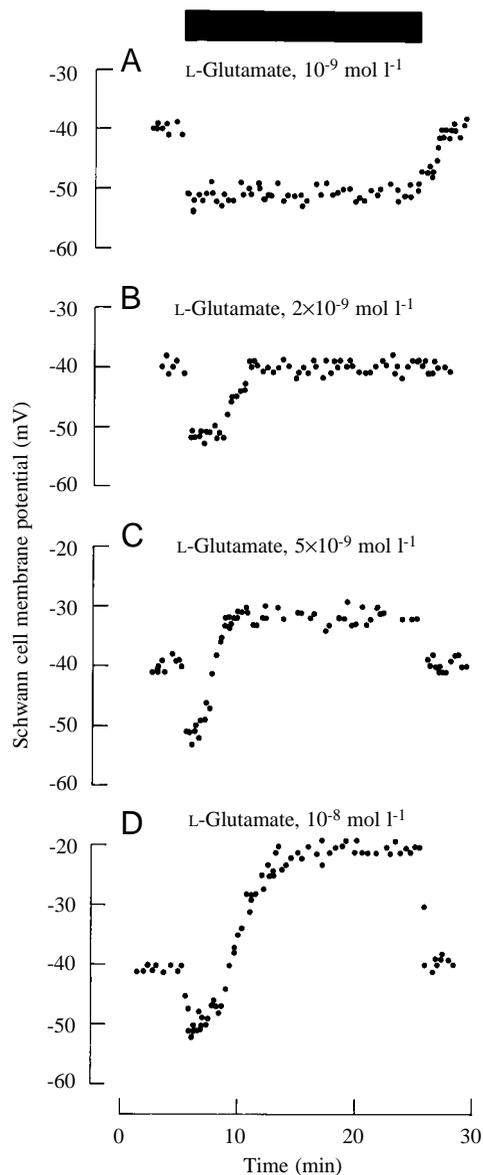


Fig. 2. (A–D) The effects of prolonged pulses (20 min) of various concentrations of L-glutamate (filled bar) on the Schwann cell membrane potential. Each point represents the potential difference recorded in a different Schwann cell.

hyperpolarizing component of the Schwann cell membrane potential due to the L-glutamate-induced release of the endogenous VIP-like peptide after blocking the cholinergic component of the response by exposure of the preparation to α -bungarotoxin, an irreversible inhibitor of the nicotinic cholinergic receptors in this preparation (Villegas, 1975). Under these conditions, a direct hyperpolarizing action of the endogenously released peptide can be demonstrated by potentiating its effects in the presence of subthreshold doses of DL-octopamine ($10^{-8} \text{ mol l}^{-1}$; Fig. 1B,C). Fig. 1C shows that the peptide-induced effect is dose-dependent and has a threshold between 2×10^{-10} and $5 \times 10^{-10} \text{ mol l}^{-1}$ for 1 min pulses of L-glutamate. This is identical to the threshold for the release of the cholinergic component. At low concentrations of

L-glutamate, the peptide response was small; at higher concentrations of L-glutamate, sufficient peptide was released to produce very large hyperpolarizations of the Schwann cell membrane potential when potentiated by DL-octopamine (Fig. 1B,C).

The release of a VIP-like peptide by prolonged glutamate pulses

Exposure of squid Schwann cells to prolonged pulses of L-glutamate has provided evidence for the presence of a number of N-methyl-D-aspartate (NMDA) glutamate receptor subtypes on these cells as well as evidence for plasticity in the responses of the Schwann cells to the application of L-glutamate after the activation of one class of NMDA receptor (Evans et al., 1991a, 1992). Thus, we have investigated the relationship between the release of acetylcholine and of the endogenous VIP-like peptide during and after exposure of the squid giant axon–Schwann cell preparation to prolonged pulses of L-glutamate.

Prolonged pulses (20 min) of L-glutamate produce a variety of concentration-dependent effects on the membrane potential of the Schwann cell of the squid giant axon. Fig. 2 shows that a pulse of $10^{-9} \text{ mol l}^{-1}$ L-glutamate induced a prolonged hyperpolarization ($N=8$), whilst a similar pulse of $2 \times 10^{-9} \text{ mol l}^{-1}$ L-glutamate induced a rapid hyperpolarization followed by a return to control levels during the course of the pulse ($N=5$). Exposure to prolonged pulses of higher concentrations (i.e. 5×10^{-9} and $10^{-8} \text{ mol l}^{-1}$) produced similar transient hyperpolarizations followed by dose-dependent slow depolarizations ($N=10$). The latter have previously been shown to be due to the activation of one class of NMDA-type glutamate receptor since they can be specifically blocked by DL-2-amino-5-phosphophonovaleric acid (APV or AP5), a selective NMDA receptor antagonist, but not by DL-2-amino-4-phosphonobutyric acid (APB) (Evans et al., 1991a, 1992).

However, lower prolonged doses of L-glutamate can also be shown to activate the NMDA receptors if given in the presence of subthreshold doses of DL-octopamine. Fig. 3A shows that a 20 min pulse of $10^{-9} \text{ mol l}^{-1}$ L-glutamate given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine produced a transient hyperpolarization followed by a return to resting membrane potential during the course of the pulse and that subsequent short pulses (1 min) of $10^{-9} \text{ mol l}^{-1}$ L-glutamate produced transient depolarizations. This suggests that the subthreshold dose of octopamine can also potentiate the activation of the NMDA receptors, as indicated by the transient reversal of the responses to 1 min pulses of L-glutamate to depolarizing responses. The latter depolarizing effects have been shown to be due to the activation of a second NMDA receptor subtype that has a different pharmacology from that responsible for the production of the slow depolarizing responses described above (Evans et al., 1992). Under the conditions used in this experiment, the prolonged L-glutamate-induced response will again be a mixed response. It represents a combination of a cholinergic response due to the release of acetylcholine, a peptide-potentiating response due to the release of the

endogenous VIP-like peptide and the response due to the activation of the NMDA-type glutamate receptors. The cholinergic component of this response can be blocked irreversibly by repeating the above experiment in the presence of $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin. Fig. 3B shows that, under these conditions, the hyperpolarizing response to the long glutamate pulse in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine

is smaller and shorter. Surprisingly, after the prolonged L-glutamate exposure, short pulses (1 min) of $10^{-9} \text{ mol l}^{-1}$ L-glutamate, even given in the presence of DL-octopamine ($10^{-8} \text{ mol l}^{-1}$), did not produce rapid depolarizations. This suggests that, after exposure to α -bungarotoxin, a prolonged pulse (20 min) of L-glutamate at a concentration of $10^{-9} \text{ mol l}^{-1}$ does not lead to the activation of either the cholinergic

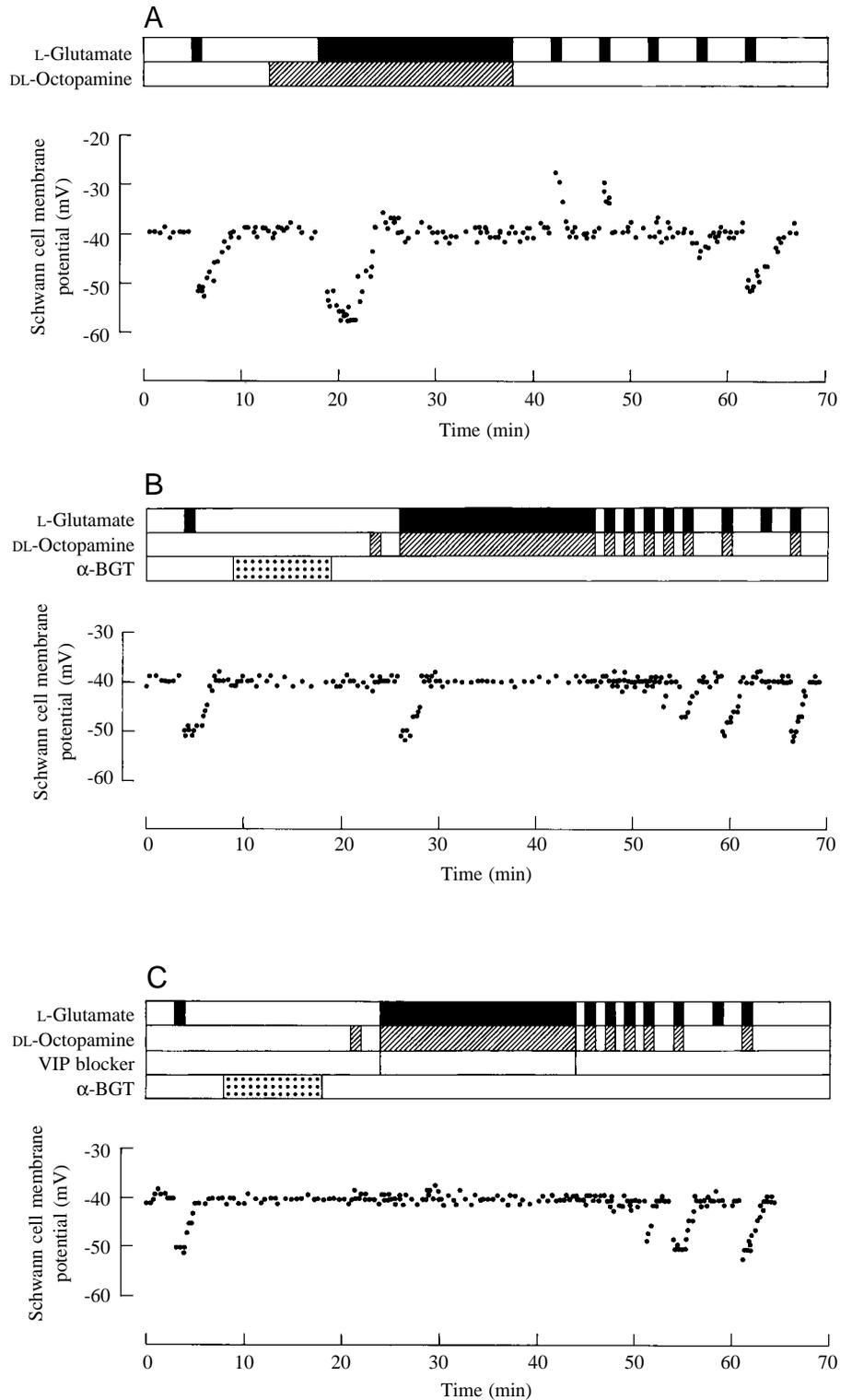


Fig. 3. The effects of a prolonged pulse (20 min) and subsequent short pulses (1 min) of $10^{-9} \text{ mol l}^{-1}$ L-glutamate (filled bars) on the Schwann cell membrane potential. (A) With the prolonged pulse given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bar). (B) After exposure to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (α -BGT) (stippled bar) and with the prolonged and short pulses given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bars). (C) As in B, except that the prolonged pulse is also given in the presence of $10^{-9} \text{ mol l}^{-1}$ of the VIP blocking agent (pCl-D-Phe⁶, Leu¹⁷)VIP (open bar). Each point represents the potential difference recorded in a different Schwann cell.

receptors or the NMDA receptors. Further, this suggests that the transient hyperpolarization induced by the prolonged L-glutamate pulse under these conditions could be due exclusively to the released VIP-like peptide. This suggestion is strongly supported by the observation that hyperpolarization

can be blocked if the prolonged pulse of L-glutamate ($10^{-9} \text{ mol l}^{-1}$) and DL-octopamine ($10^{-8} \text{ mol l}^{-1}$) is given in the presence of a VIP blocker ($10^{-9} \text{ mol l}^{-1}$) (Fig. 3C). Thus, a prolonged pulse of $10^{-9} \text{ mol l}^{-1}$ L-glutamate gives rise to a prolonged release of acetylcholine that lasts as long as

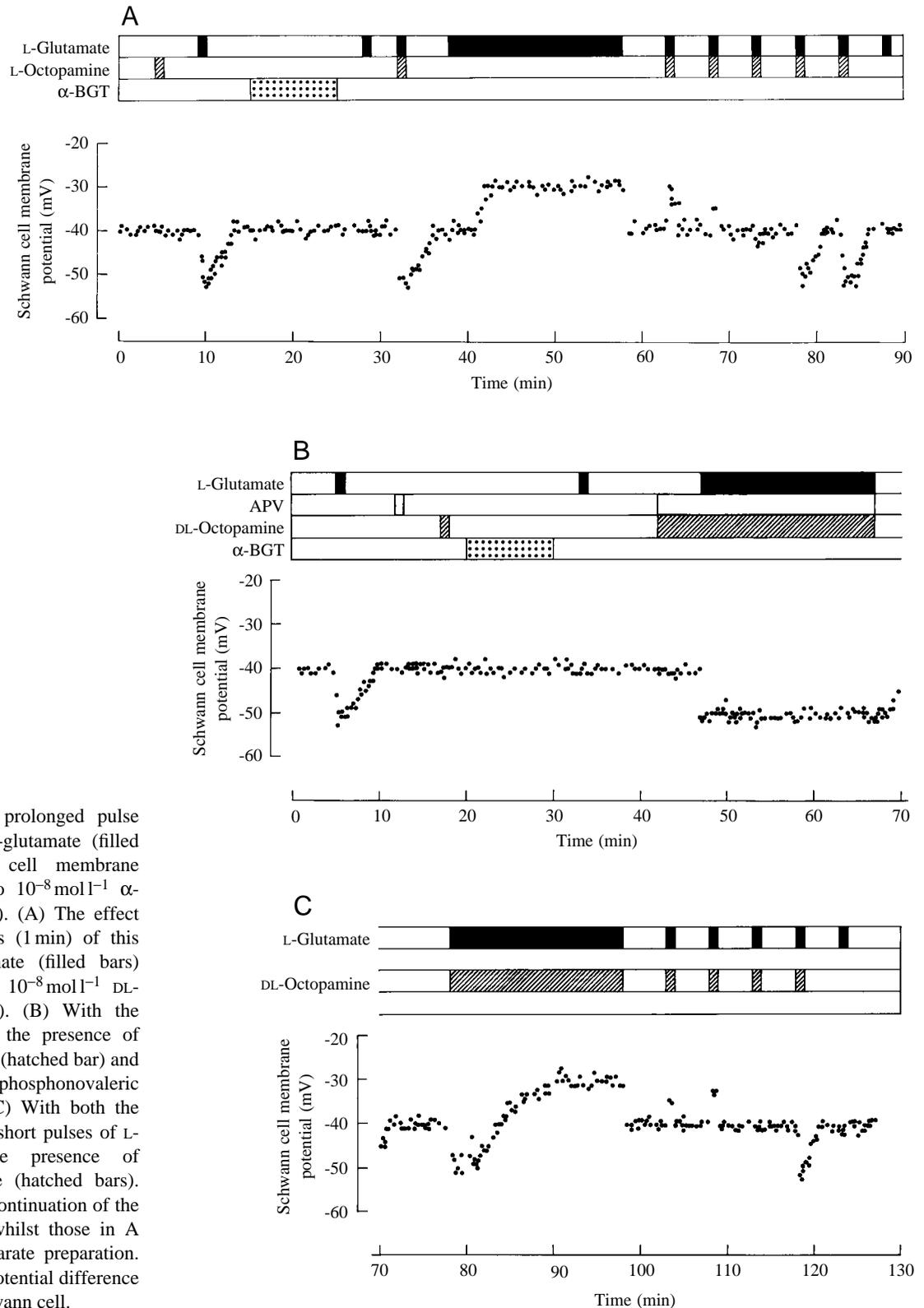


Fig. 4. The effects of a prolonged pulse (20 min) of $10^{-8} \text{ mol l}^{-1}$ L-glutamate (filled bars) on the Schwann cell membrane potential after exposure to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (stippled bar). (A) The effect of subsequent short pulses (1 min) of this concentration of L-glutamate (filled bars) given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bars). (B) With the prolonged pulse given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bar) and $10^{-6} \text{ mol l}^{-1}$ DL-2-amino-5-phosphonovaleric acid (APV) (open bar). (C) With both the prolonged and subsequent short pulses of L-glutamate given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bars). The recordings in C are a continuation of the experiment shown in B, whilst those in A were obtained from a separate preparation. Each point represents the potential difference recorded in a different Schwann cell.

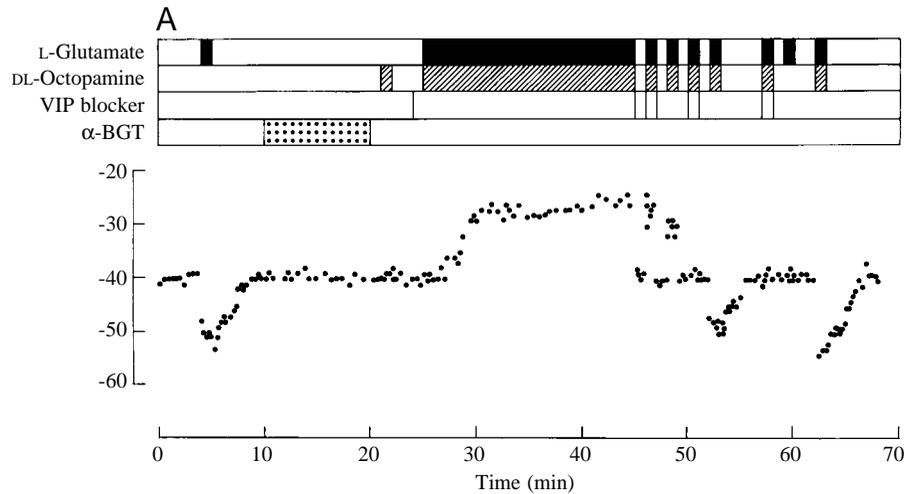
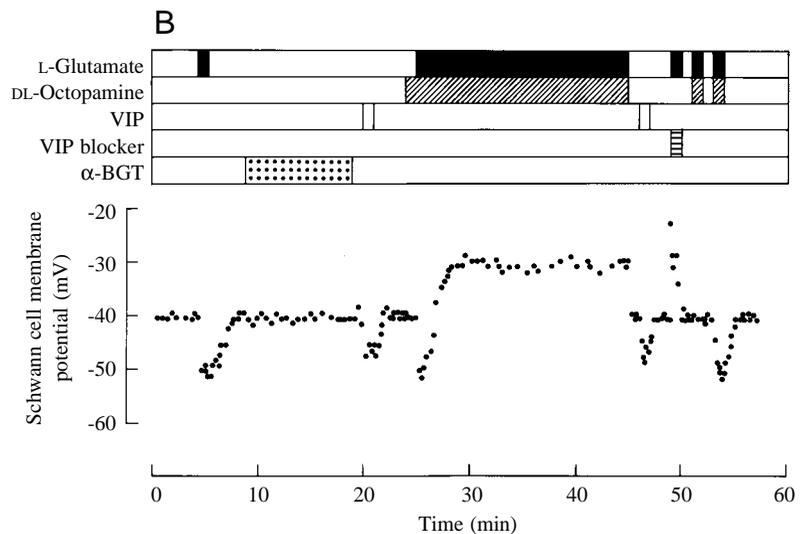


Fig. 5. The effects of a prolonged pulse (20 min) and subsequent short pulses (1 min) of $10^{-8} \text{ mol l}^{-1}$ L-glutamate (filled bars) on the Schwann cell membrane potential after exposure to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (α -BGT) (stippled bar). (A) With the prolonged and some of the short pulses given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bars) and/or the VIP-blocking agent (pCl-D-Phe⁶, Leu¹⁷)VIP ($10^{-9} \text{ mol l}^{-1}$) (open bars). (B) With the prolonged and some of the short pulses given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bars) and one of the short pulses in the presence of the VIP-blocking agent alone (horizontally striped bar). In addition, this experiment shows the effect of a short pulse (1 min) of $10^{-9} \text{ mol l}^{-1}$ VIP (open bar) given immediately after the prolonged pulse. Each point represents the potential difference recorded in a different Schwann cell.



glutamate is applied, but only to a short transient release of the VIP-like peptide lasting a couple of minutes.

Fig. 3B,C also indicates that, after a prolonged pulse (20 min) of $10^{-9} \text{ mol l}^{-1}$ L-glutamate in a preparation that has been exposed to α -bungarotoxin ($10^{-8} \text{ mol l}^{-1}$) to block irreversibly the cholinergic receptors, the short hyperpolarizing responses due to the release of the VIP-like peptide by 1 min pulses of L-glutamate ($10^{-9} \text{ mol l}^{-1}$) in the presence of DL-octopamine ($10^{-8} \text{ mol l}^{-1}$) do not start to recover until 10–15 min after the end of the prolonged pulse. This is a similar time course to the recovery of the L-glutamate-induced cholinergic responses (Evans et al., 1991a, 1992).

The lack of activation of the NMDA receptors by a prolonged pulse of $10^{-9} \text{ mol l}^{-1}$ L-glutamate after exposure of the preparation to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin can be overcome by increasing the concentration of L-glutamate in the pulse to $10^{-8} \text{ mol l}^{-1}$ (Fig. 4A). Under these circumstances, the cholinergic receptors are still blocked, but the prolonged L-glutamate pulse produces a slow depolarization due to the activation of one class of NMDA receptor and rapid

depolarizations in response to short pulses (1 min) of $10^{-8} \text{ mol l}^{-1}$ L-glutamate at the end of the prolonged pulse due to the subsequent activation of the second class of NMDA receptor. If the prolonged pulse of $10^{-8} \text{ mol l}^{-1}$ L-glutamate is given in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine, the response is converted to a transient hyperpolarization followed by a slow depolarization (Fig. 4C). The slow depolarization is due to the activation of the NMDA receptors since the response can be converted to a prolonged hyperpolarization in the presence of APV ($10^{-6} \text{ mol l}^{-1}$) (Fig. 4B). The latter prolonged hyperpolarization elicited under these conditions can be shown to be produced by the release of the endogenous VIP-like peptide since the biphasic response described above (Fig. 4C) can be converted into a slow depolarization in the presence of the VIP blocking agent (pCl-D-Phe⁶ Leu¹⁷)VIP ($10^{-9} \text{ mol l}^{-1}$) (Fig. 5A).

The above results suggest that the time course of the hyperpolarizing effect produced in the Schwann cells due to the release of the endogenous VIP-like peptide is dependent on the concentration of L-glutamate in the prolonged pulse. It also

suggests that activation of the NMDA receptors by the prolonged pulse of $10^{-8} \text{ mol l}^{-1}$ L-glutamate is not dependent upon the activation of the VIP receptor in this preparation. In addition, the experiment shown in Fig. 5A indicates that the rapid depolarizations initiated by short pulses (1 min) of $10^{-8} \text{ mol l}^{-1}$ L-glutamate due to the activation of the second

class of NMDA receptor immediately after the prolonged depolarization induced by the first class of NMDA receptor (Evans et al., 1991a, 1992) are not dependent on the release of the VIP-like peptide since they are not blocked by the VIP blocking agent. However, the transient hyperpolarizations that develop 10–15 min after the end of the NMDA-induced slow

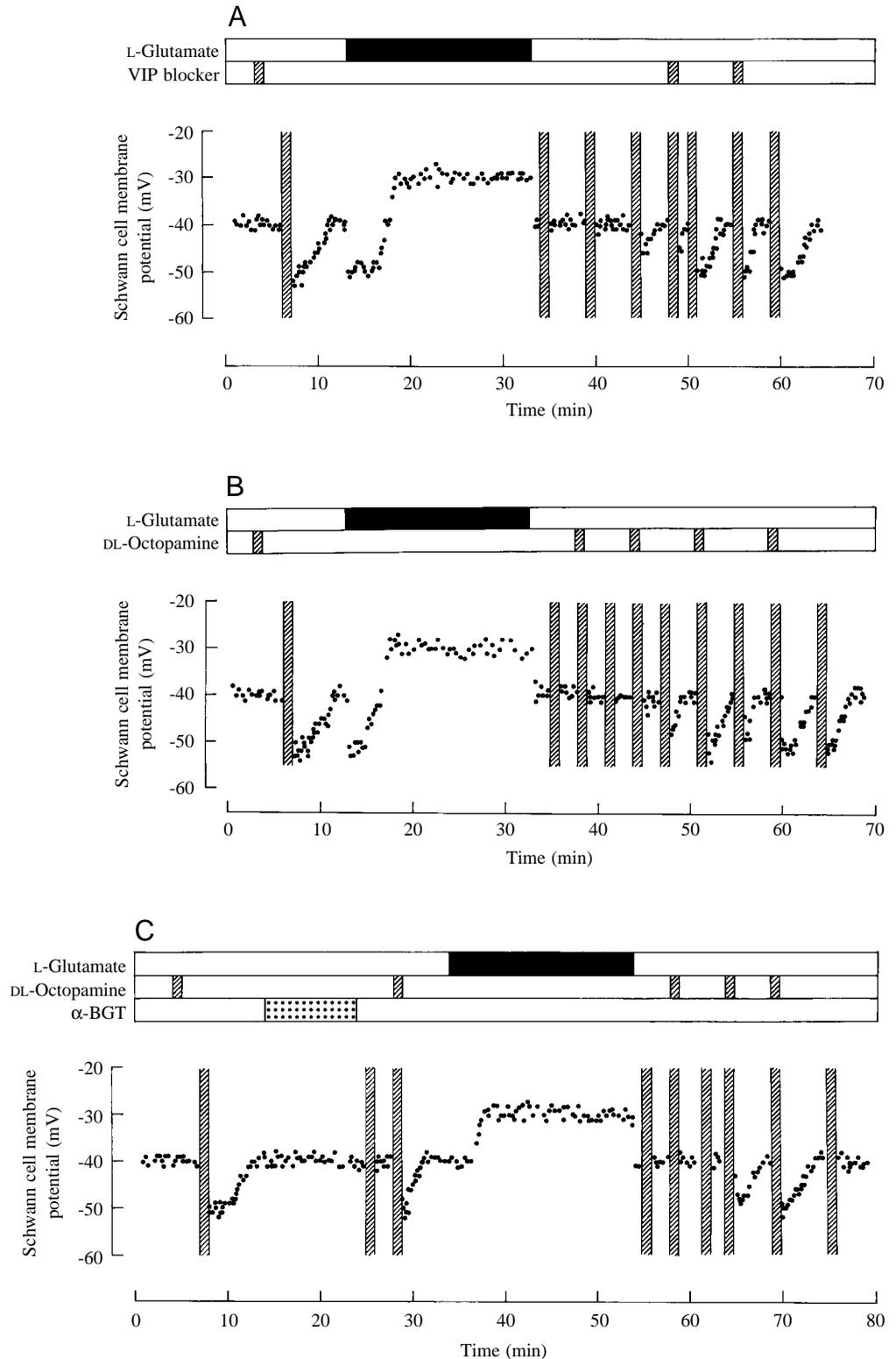


Fig. 6. The effects of a prolonged pulse (20 min) of $10^{-8} \text{ mol l}^{-1}$ L-glutamate (filled bar) on the response of the Schwann cell membrane potential to stimulation of the giant axon at 100 Hz for 1 min (vertical hatched bars). (A) With axonal stimulation given in the presence and absence of the VIP-blocking agent (pCl-D-Phe⁶, Leu¹⁷)VIP ($10^{-9} \text{ mol l}^{-1}$) (hatched bars). (B) With axonal stimulation given in the presence and absence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (hatched bars). (C) As in B, except that the preparation was pre-exposed to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (α -BGT) (stippled bar). Each point represents the potential difference recorded in a different Schwann cell.

depolarization in response to short pulses (1 min) of $10^{-8} \text{ mol l}^{-1}$ L-glutamate in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine are due to the release of the VIP-like peptide since they are blocked by the VIP blocking agent (Fig. 5A). The slow development of these responses is due to the recovery of the release of the VIP-like peptide after blockage of the release mechanism due to the activation of the NMDA receptors. The slow development of these responses is not due to a change in the responsiveness of the preparation to exogenously applied VIP, since a short pulse (1 min) of $10^{-9} \text{ mol l}^{-1}$ VIP is capable of inducing a transient hyperpolarization when given immediately after the end of a slow NMDA-receptor-induced depolarization (Fig. 5B).

The release of a VIP-like peptide after axonal stimulation

In the experiments described above, the recovery of the release of acetylcholine and the VIP-like peptide after exposure of the preparation to a prolonged pulse of L-glutamate, at a concentration high enough to activate the NMDA-type glutamate receptors, was monitored by the application of short pulses (1 min) of L-glutamate. Similar results were obtained if the recovery was assessed by comparing the responses of the Schwann cells to short periods (1 min) of axonal stimulation at 100 Hz (Fig. 6). Fig. 6A indicates that, after a slow depolarization initiated by a 20 min pulse of $10^{-8} \text{ mol l}^{-1}$ L-glutamate, short periods (1 min) of axonal stimulation at 100 Hz produced no immediate effect on the Schwann cell membrane potential. However, after 10–15 min, such periods of axonal stimulation again induced rapid hyperpolarizing responses which could be reduced in size and time course when given in the presence of the VIP blocking agent (pCl-D-Phe⁶, Leu¹⁷)VIP ($10^{-9} \text{ mol l}^{-1}$), indicating that they represent cholinergic responses that have been potentiated by the simultaneous release of the endogenous VIP-like peptide. Similarly, after such a slow depolarization, axonal stimulation, even in the presence of low doses of DL-octopamine, did not initiate any responses for 10–15 min (Fig. 6B). When the responses did return, they were potentiated by octopamine as demonstrated previously (Evans et al., 1986). The recovery of the release of the endogenous VIP-like peptide after a prolonged glutamate pulse can be followed in experiments in which the cholinergic responses are blocked after exposure of the preparation to $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (Fig. 6C). Here, stimulation at 100 Hz in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine prior to the initiation of the glutamate-induced slow depolarization can be used to demonstrate the release of the endogenous VIP-like peptide (Evans et al., 1986). However, no response could be demonstrated until 10–15 min after the end of the slow depolarization induced by activation of the NMDA receptors, which is the same time taken for the recovery of the cholinergic responses in this preparation.

Discussion

Information about the activity level of the squid giant axon is signalled to the adaxonal Schwann cells by the complex

Table 1. A schematic outline of the steps involved in signalling squid giant axon activity to the surrounding adaxonal Schwann cells

1	Glutamate release from the activated giant axon into the adaxonal space
2	Glutamate activates specific receptors and/or a transporter on Schwann cells
3	Induction of co-release (?) of acetylcholine and VIP-like peptide from Schwann cells by a Ca^{2+} -dependent process
4	Acetylcholine and VIP-like peptide feed back to activate specific autoreceptors on Schwann cells
5	Nicotinic acetylcholine receptor activation leads to Schwann cell membrane hyperpolarization via a second-messenger-mediated pathway; VIP-like peptide receptor activation potentiates the actions of the cholinergic pathway
6	Schwann cells receive information about giant axon activity

VIP, vasoactive intestinal peptide.

multistep pathway summarised in Table 1. In the present study, we have shown that a VIP-like peptide is released both by axonal stimulation and by the application of L-glutamate to the giant axon–Schwann cell preparation from the tropical squid *Sepioteuthis sepioidea*. The release of this peptide closely parallels the previously described release of acetylcholine from the Schwann cells in this preparation (Villegas, 1973, 1975; Villegas and Jenden, 1979). First, the release of both molecules occurs with the same threshold of between 2×10^{-10} and $5 \times 10^{-10} \text{ mol l}^{-1}$ for 1 min applications of L-glutamate. Second, exposure of preparations to prolonged pulses of L-glutamate leads to the activation of a class of NMDA-type glutamate receptor which results in a block of the release of both acetylcholine and the VIP-like peptide. Third, after such a blockage, the recoveries of the release of both acetylcholine and the VIP-like peptide have the same time course.

The simplest explanation for the above results is that the VIP-like peptide and the acetylcholine are co-released from the Schwann cells after activation of the metabotropic quisqualate/kainate subtype of glutamate receptors (Evans et al., 1992) either by externally applied L-glutamate or by glutamate released from the giant axon upon stimulation. However, the present results cannot directly rule out the possibility that the exogenously applied, or endogenously released, glutamate in some way feeds back onto the giant axon to initiate a release of the VIP-like peptide whilst acting on the Schwann cells to release acetylcholine. Nonetheless, the latter explanation seems unlikely since it would require both the giant axon and the Schwann cells to have quisqualate/kainate-subtype glutamate receptors with the same thresholds for the activation of release. In addition, both cell types would also have to possess identical NMDA-type glutamate receptors and, after their activation, acetylcholine release from the Schwann cells and the release of the VIP-like peptide from the giant axon would need to be blocked and to recover with identical time

courses. Evidence is lacking for the existence of glutamate receptors on non-synaptic regions of the squid giant axon. In a number of studies, the application of L-glutamate to non-synaptic regions of the squid giant axon has not been shown to alter its resting potential or the shape of action potentials (Messenger et al., 1995). Also, glutamate application does not alter the levels of cyclic AMP (Baker and Carruthers, 1984) or cyclic GMP (Allen and Rouot, 1995) in squid giant axons. Thus, the hypothesis we favour is that the acetylcholine and the VIP-like peptide are likely to be co-released from the Schwann cells. Nevertheless, it should be noted that we are not able, with the techniques currently available, to rule out the possibility that different subpopulations of Schwann cells are responsible for the release of acetylcholine and the release of the VIP-like peptide.

Experiments with prolonged (20 min) pulses of L-glutamate indicate the possibility that the release of acetylcholine and the VIP-like peptide in this preparation may be differentially sensitive to the level of intracellular Ca^{2+} . Prolonged pulses of $10^{-8} \text{ mol l}^{-1}$ L-glutamate given in the presence of DL-octopamine ($10^{-8} \text{ mol l}^{-1}$) and APV ($10^{-6} \text{ mol l}^{-1}$), after pretreatment of the preparation with $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin, induce a sustained release of the VIP-like peptide for the duration of the L-glutamate pulse, in a fashion similar to the release of acetylcholine from the Schwann cells (Villegas, 1981; Evans et al., 1991a). This experiment also indicates that the release of the VIP-like peptide is not dependent upon the activation of the NMDA receptors or the nicotinic cholinergic receptors and is likely to be mediated *via* the activation of the metabotropic subclass of L-glutamate receptors on the Schwann cells (Evans et al., 1992). However, if this experiment is repeated with a pulse of L-glutamate at a concentration of $10^{-9} \text{ mol l}^{-1}$, the results are slightly different. Under these conditions, the release of acetylcholine still persists for the duration of the prolonged L-glutamate pulse, but in the presence of α -bungarotoxin ($10^{-8} \text{ mol l}^{-1}$) the release of the VIP-like peptide is now only transient (2–3 min) and disappears even though L-glutamate is still present. In this experiment, it is not clear why the presence of α -bungarotoxin blocks the activation of both the cholinergic and the NMDA receptors. It is possible that, under these conditions, the potentiating effect of DL-octopamine, which is mediated *via* an elevation of cyclic AMP concentration (Reale et al., 1986), has an additive effect with the increased cyclic AMP levels produced by activation of the nicotinic cholinergic receptors (Evans et al., 1985) and that the removal of the latter cholinergic component in the presence of α -bungarotoxin does not provide sufficient potentiation for this concentration of L-glutamate to activate the NMDA receptors. We have previously hypothesised that activation of the metabotropic glutamate receptors may lead to a release of acetylcholine as the result of an elevation in Schwann cell Ca^{2+} levels (Evans et al., 1992, 1995). Thus, it is possible that the release of the VIP-like peptide and of acetylcholine from the Schwann cells could be differentially sensitive to intracellular Ca^{2+} levels. In neurones, it has been suggested that small elevations in the

Ca^{2+} concentration in the bulk cytoplasm are required for a non-synaptic release of neuropeptides, in contrast to the need to produce greater increases in Ca^{2+} concentration at specific synaptic sites in the vicinity of Ca^{2+} channels for the release of conventional neurotransmitters (Verhage et al., 1991). Further studies are needed on the release mechanisms of peptidergic and non-peptidergic neurotransmitters from glial cells to explain the mechanisms underlying the above putative co-release process.

It is clear from the results of the present study that activation of the receptors for the endogenously released VIP-like peptide is not required for activation of either of the two subclasses of NMDA receptor previously described to be present in this preparation (Evans 1991a, 1992). In addition, a short pulse of VIP can initiate an immediate transient hyperpolarization of the Schwann cells after a prolonged pulse of L-glutamate has activated the NMDA receptors, whereas a short pulse of glutamate or a 1 min period of axonal stimulation at 100 Hz in the presence of a subthreshold dose of octopamine produces no hyperpolarization for a period of 10–15 min after the end of the prolonged L-glutamate pulse. This suggests that the release of the endogenous VIP-like peptide is blocked after exposure of the preparation to a prolonged pulse of L-glutamate in a fashion similar to the release of acetylcholine (Villegas, 1981; Evans et al., 1992). The results of the present study indicate that the recovery of the release of acetylcholine and the release of the endogenous VIP-like peptide from the Schwann cell have the same time course after being blocked by exposure to a prolonged pulse of L-glutamate. At present, the mechanism of this inhibition of their release is not known, but it would appear to involve an independent mechanism from the activation of the NMDA receptors responsible for the fast depolarizing responses seen transiently after exposure to a prolonged pulse of L-glutamate.

Although a considerable amount of information suggests that glial cells may release a range of neuroactive substances (Martin, 1992), very little is known about the functional roles of such released material. This is largely because most of the work has been carried out on astrocyte cultures in the absence of specific target sites. In addition, in many cases, it is not clear whether the released material has been synthesised by the cells that release it or has been actively accumulated by specific uptake mechanisms. However, at the denervated frog neuromuscular junction, Schwann cells release multimolecular amounts of acetylcholine that evoke miniature endplate potentials in the muscle (Dennis and Miledi, 1974; Ito and Miledi, 1977). In a similar rat preparation, the release of acetylcholine occurs only as a specific response to denervation (Brockes, 1984) and may serve in some way to maintain the postsynaptic integrity of the synaptic site until it can be reoccupied by a regenerating motor nerve terminal.

Although the presence of VIP-like peptides has been reported in astrocytoma cell lines (Said and Rosenberg, 1976) and in non-neuronal fractions of brain tissue (Besson et al., 1979; see Rostène, 1984), to our knowledge a co-release of a VIP-like peptide and acetylcholine has not been reported from any glial

cell preparation, although it has been reported from neurones innervating the cat submandibular salivary gland (Lundberg, 1981; Lundberg et al., 1982). This raises the question of the functional role of the release of these two substances from the Schwann cells surrounding the squid giant axon. It would appear that in the animal, where the firing pattern of the giant axon is likely to be slower and shorter than the 100 Hz test pulses for 1 min used in the present study, the quantity of VIP-like peptide released will be small and insufficient to produce any direct effects on the Schwann cell membrane potential. Its functional role is therefore likely to be to potentiate both the amplitude and time course of the hyperpolarizations in the Schwann cell membrane potential produced by the released acetylcholine acting on the nicotinic cholinergic receptors (Evans et al., 1986). However, it is also possible that the released VIP-like neuropeptide could have other actions, for instance to stimulate the breakdown of metabolic reserves in the Schwann cells, which would not have been detected in the present experiments. Since the actions of both the cholinergic and peptidergic components of this co-release system can be potentiated independently in the presence of low concentrations of DL-octopamine, which may act as a circulatory hormone released into squid haemolymph under stressful conditions (Reale et al., 1986), this system may have evolved to give the animal maximal flexibility in the use of this signalling system. This conclusion is further supported by the idea that the cholinergic and peptidergic components of the release system may be under independent regulation under some circumstances.

At present, the functional significance of the signalling pathway between the squid giant axon and the Schwann cells is unknown. It has been suggested that it could function in the spatial buffering of K^+ released into the adaxonal space around the giant axon at times of high activity (e.g. see Abbott et al., 1995). In addition, it is also possible that the signal could function to generate a metabolic response from the Schwann cells at times of high axonal activity. It is interesting to note in the latter context that the release of VIP, amongst other modulators, in the mammalian cortex has been implicated in the generation of spatial domains for the control of energy metabolism (see Pentreath et al., 1986) and that VIP can initiate glycogenolysis in cultured astrocytes from neonatal rat brain (Magistretti et al., 1983). Further studies are required to determine whether VIP-like peptides serve a similar functional role in the complex process of signalling between the giant axon and the Schwann cells in the squid nervous system.

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References

- Abbott, N. J., Brown, E. R., Pichon, Y. and Kukita, F. (1995). Electrophysiology of squid Schwann cells. In *Cephalopod Neurobiology* (ed. N. J. Abbott, R. Williamson and L. Maddock), pp. 198–212. Oxford: Oxford University Press.
- Allen, T. J. A. and Rouot, B. (1995). Cyclic nucleotide homeostasis and axonal G proteins in the squid *Loligo forbesi*. In *Cephalopod Neurobiology* (ed. N. J. Abbott, R. Williamson and L. Maddock), pp. 35–52. Oxford: Oxford University Press.
- Baker, P. F. and Carruthers, A. (1984). Hormone-sensitive cyclic nucleotide metabolism in giant axons of *Loligo*. *Curr. Topics Membr. Transport* **22**, 271–276.
- Bartfai, T., Iverfeldt, K., Fisone, G. and Serfozo, P. (1988). Regulation of the release of coexisting transmitters. *Annu. Rev. Pharmacol. Toxicol.* **28**, 285–310.
- Besson, J., Rotsztein, W., Laburthe, M., Epelbaum, J., Beaudet, A., Kordon, C. and Rosselin, G. (1979). Vasoactive Intestinal Peptide (VIP): Brain distribution, subcellular localization and effect of deafferentation of the hypothalamus, in male rats. *Brain Res.* **165**, 79–86.
- Brockes, J. P. (1984). Assays for cholinergic properties in cultured rat Schwann cells. *Proc. R. Soc. Lond. B* **222**, 121–134.
- Brown, E. R. and Abbott, N. J. (1992). Membrane potential in squid Schwann cells: stable impalements in the split open giant axon preparation of *Loligo*. *J. Physiol., Lond.* **446**, 320P.
- Brown, E. R., Bone, Q., Ryan, K. P. and Abbott, N. J. (1991). Morphology and electrical properties of Schwann cells around the giant axon of the squids *Loligo forbesi* and *Loligo vulgaris*. *Proc. R. Soc. Lond. B* **243**, 255–262.
- Brunder, D. G. and Lieberman, E. M. (1988). Studies of axon–glial cell interactions and periaxonal K^+ homeostasis. I. The influence of Na^+ , K^+ , Cl^- and cholinergic agents on the membrane potential of the adaxonal glia of the crayfish medial giant axon. *Neuroscience* **25**, 951–959.
- Church, P. J., Whim, M. D. and Lloyd, P. E. (1993). Modulation of neuromuscular transmission by conventional and peptide transmitters released from excitatory and inhibitory motor neurons in *Aplysia*. *J. Neurosci.* **13**, 2790–2800.
- Dennis, M. J. and Miledi, R. (1974). Electrically induced release of acetylcholine from denervated Schwann cells. *J. Physiol., Lond.* **237**, 431–452.
- Evans, P. D., Reale, V., Merzon, R. M. and Villegas, J. (1990). Substance P modulation of the membrane potential of the Schwann cell of the squid giant nerve fibre. *Glia* **3**, 393–404.
- Evans, P. D., Reale, V., Merzon, R. M. and Villegas, J. (1991a). *N*-methyl-D-aspartate (NMDA) and non-NMDA type glutamate receptors are present on squid giant axon Schwann cells. *J. Exp. Biol.* **157**, 593–600.
- Evans, P. D., Reale, V., Merzon, R. M. and Villegas, J. (1991b). Mechanisms of axon–Schwann cell signalling in the squid nerve fibre. In *Glial–Neuronal Interactions*, vol. 633 (ed. N. J. Abbott), pp. 434–447. Annals of the New York Academy of Science.
- Evans, P. D., Reale, V., Merzon, R. M. and Villegas, J. (1992). *N*-methyl-D-aspartate (NMDA) and non-NMDA type glutamate receptors modulate the properties of the Schwann cell of the squid giant nerve. *J. Exp. Biol.* **173**, 229–249.
- Evans, P. D., Reale, V., Merzon, R. M. and Villegas, J. (1995). The pharmacology of receptors present on squid giant axon Schwann cells. In *Cephalopod Neurobiology* (ed. N. J. Abbott, R. Williamson and L. Maddock), pp. 213–228. Oxford: Oxford University Press.
- Evans, P. D., Reale, V. and Villegas, J. (1985). The role of cyclic nucleotides in modulation of the membrane potential of the

- Schwann cell of squid giant nerve fibre. *J. Physiol., Lond.* **363**, 151–167.
- Evans, P. D., Reale, V. and Villegas, J.** (1986). Peptidergic modulation of the membrane potential of the Schwann cell of the squid giant nerve fibre. *J. Physiol., Lond.* **379**, 61–82.
- Evans, P. D. and Villegas, J.** (1988). The action of vasoactive intestinal peptide antagonists on peptidergic modulation of the squid Schwann cell. *J. Exp. Biol.* **138**, 259–269.
- Hökfelt, T. O., Millhom, D., Seroogy, K., Tsuruo, Y., Ceccatelli, S., Lindh, B., Meister, B., Melander, T., Schalling, M., Bartfai, T. and Terenius, L.** (1987). Co-existence of peptides with classical neurotransmitters. *Experientia* **43**, 768–780.
- Ito, Y. and Miledi, R.** (1977). The effect of calcium-ionophores on acetylcholine release from Schwann cells. *Proc. R. Soc. Lond. B* **196**, 51–58.
- Iversen, L. L.** (1984). Amino acids and peptides: fast and slow chemical signals in the nervous system. *Proc. R. Soc. Lond. B* **221**, 245–260.
- Kupfermann, I.** (1991). Functional studies of cotransmission. *Physiol. Rev.* **71**, 683–732.
- Lieberman, E. M., Abbott, N. J. and Hassan, S.** (1989). Evidence that glutamate mediates axon-to-Schwann cell signaling in the squid. *Glia* **2**, 94–102.
- Lieberman, E. M., Hargittai, P. T. and Grossfeld, R. M.** (1994). Electrophysiological and metabolic interactions between axons and glia in crayfish and squid. *Prog. Neurobiol.* **44**, 333–376.
- Lieberman, E. M. and Sanzenbacher, E.** (1992). Mechanisms of glutamate activation of axon-to-Schwann cell signaling in the squid. *Neuroscience* **47**, 931–939.
- Lundberg, J. M.** (1981). Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. *Acta Physiol. Scand.* (Suppl. 496) **112**, 1–57.
- Lundberg, J. M., Anggard, A., Fahrenkrug, J., Lundgren, G. and Holmstedt, B.** (1982). Co-release of VIP and acetylcholine in relation to blood flow and saliva secretion in cat submandibular salivary gland. *Acta Physiol. Scand.* **115**, 525–528.
- Magistretti, P. J., Manthorpe, M., Bloom, F. E. and Varon, S.** (1983). Functional receptors for vasoactive intestinal polypeptide in cultured astroglia from neonatal rat brain. *Regul. Peptides* **6**, 71–80.
- Martin, D. L.** (1992). Synthesis and release of neuroactive substances by glial cells. *Glia* **5**, 81–94.
- Messenger, J. B.** (1996). Neurotransmitters of cephalopods. *Invertebrate Neurosci.* **2**, 95–114.
- Messenger, J. B., De Santis, A. and Ogden, D. C.** (1995). Chemical transmission at the squid giant synapse. In *Cephalopod Neurobiology* (ed. N. J. Abbott, R. Williamson and L. Maddock), pp. 283–297. Oxford: Oxford University Press.
- Pentreath, V. W., Seal, L. H., Morrison, J. H. and Magistretti, P. J.** (1986). Transmitter mediated regulation of energy metabolism in nervous tissue at the cellular level. *Neurochem. Int.* **9**, 1–10.
- Reale, V., Evans, P. D. and Villegas, J.** (1986). Octopaminergic modulation of the membrane potential of the Schwann cell of the squid giant nerve fibre. *J. Exp. Biol.* **121**, 421–443.
- Rostène, W. H.** (1984). Neurobiological and neuroendocrine functions of the vasoactive intestinal peptide (VIP). *Prog. Neurobiol.* **22**, 103–129.
- Said, S. I. and Rosenberg, R. N.** (1976). Vasoactive intestinal polypeptide: Abundant immunoreactivity in neural cell lines and normal nervous tissue. *Science* **192**, 907–908.
- Verhage, M., McMahon, H. T., Ghijsen, W. E. J. M., Boomsma, F., Scholten, G., Wiegant, V. M. and Nicholls, D. G.** (1991). Differential release of amino acids, neuropeptides and catecholamines from isolated nerve terminals. *Neuron* **6**, 517–524.
- Villegas, J.** (1972). Axon–Schwann cell interaction in the squid nerve fibre. *J. Physiol., Lond.* **225**, 275–296.
- Villegas, J.** (1973). Effects of tubocurarine and eserine on the axon Schwann cell relationship in the squid nerve fibre. *J. Physiol., Lond.* **232**, 193–208.
- Villegas, J.** (1975). Characterization of acetylcholine receptors in the Schwann cell membrane of the squid nerve fibre. *J. Physiol., Lond.* **249**, 679–689.
- Villegas, J.** (1978a). Cholinergic properties of satellite cells in the peripheral nervous system. In *Dynamic Properties of Glia Cells* (ed. E. Schoffeniels, G. Franck, L. Hertz and D. B. Tower), pp. 207–215. Oxford: Pergamon Press.
- Villegas, J.** (1978b). Cholinergic systems in axon–Schwann cell interactions. *Trends Neurosci.* **1**, 66–68.
- Villegas, J.** (1981). Axon/Schwann cell relationships in the giant nerve fibre of the squid. *J. Exp. Biol.* **95**, 135–151.
- Villegas, J.** (1984). Axon–Schwann cell relationship. *Curr. Topics Membr. Transport* **22**, 547–571.
- Villegas, J., Evans, P. D. and Reale, V.** (1988). Electrophysiology of Schwann cell receptors. In *Glial Cell Receptors* (ed. H. K. Kimmelberg), pp. 141–157. New York: Raven Press.
- Villegas, J. and Jenden, D. J.** (1979). Acetylcholine content of the Schwann cell and axon in the giant nerve fibre of the squid. *J. Neurochem.* **32**, 761–766.