

THE ACTION OF VASOACTIVE INTESTINAL PEPTIDE ANTAGONISTS ON PEPTIDERGIC MODULATION OF THE SQUID SCHWANN CELL

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Summary

1. The effects of two specific antagonists of vasoactive intestinal peptide (VIP) receptors were investigated on the VIP-induced hyperpolarization of the membrane potential of the Schwann cell of the giant nerve fibre of the tropical squid.

2. Both (*p*Cl-D-Phe⁶,Leu¹⁷)VIP and (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide competitively and reversibly blocked the effects of VIP in this preparation with the former compound being more potent than the latter.

3. The blocking actions of both antagonists were specific for the responses of this preparation to VIP. They did not block the actions of carbachol, DL-octopamine or substance P.

4. Both antagonists also reduced the effectiveness of an endogenous VIP-like component in the normal hyperpolarizing action of giant axon activity on the membrane potential of the Schwann cell, with the same potency ratio as for their actions on the effects induced by the exogenous application of VIP.

Introduction

Vasoactive intestinal peptide (VIP) receptors are present on membranes of both glia and neurones (Rostène, 1984; Said, 1984). However, very little is known of their functional role in glial cells since most studies have concentrated on their ability to regulate the level of adenosine 3',5'-cyclic monophosphate (cyclic AMP) in primary glial cultures (Rougon *et al.* 1983; Evans *et al.* 1984; Chneweiss *et al.* 1984, 1986; Koh *et al.* 1984). Studies on the functional interactions between glial cells and neurones require the preservation of their anatomical relationships. One intact system in which this relationship is well preserved is the preparation of the giant nerve fibre of the stellate nerve of the tropical squid, *Sepioteuthis sepioidea*. The neuronal–glial cell interactions in this preparation have been well characterized (see Villegas, 1981, 1984). Both nicotinic cholinergic receptors (Evans *et al.* 1985) and octopaminergic receptors (Reale *et al.* 1986) modulate the membrane

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potential of the satellite glial Schwann cells surrounding the giant axon by mechanisms that change the intracellular levels of cyclic AMP. In addition, the membrane potential of the Schwann cell is also modulated by a variety of peptidergic receptors. These include a class of receptors that has many pharmacological similarities with VIP receptors described in other preparations (Evans *et al.* 1986). Evidence has also been presented in this preparation for the involvement of an endogenous VIP-like peptide in the functional interactions between the giant axon and the Schwann cells (Evans *et al.* 1986). The actions of both exogenously applied VIP and the endogenous VIP-like peptide in this preparation are blocked in the presence of low concentrations of avian pancreatic polypeptide (APP). However, the mechanism of this functional inhibition is not known. APP has also been reported to block some, but not all, of the effects of VIP in a number of other preparations, but again the mode of action remains unclear (Lundberg, 1981; Fredholm & Lundberg, 1982; Karpinski *et al.* 1984). The case for the existence of authentic VIP receptors on the squid Schwann cell membrane and for the existence of an endogenous VIP-like peptide involved in the normal signalling process between the giant axon and the Schwann cell would be considerably strengthened by the application of antagonists shown to be specific for VIP receptors in other preparations.

The present paper describes the actions of two specific competitive antagonists of VIP receptors, (*p*Cl-D-Phe⁶,Leu¹⁷)VIP (Pandolf *et al.* 1986) and (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide (Waelbroeck *et al.* 1985), on the effects of exogenously applied VIP on the membrane potential of the squid Schwann cell and on the effects produced by the endogenous release of a VIP-like peptide in the same preparation.

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). Electrophysiological techniques were as described previously and involved the successive measurement of electrical potentials of a series of Schwann cells by brief impalements from inside the axon (Villegas, 1972, 1973, 1975). All experiments were carried out at room temperature (20–22°C).

Drugs which were 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} 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 95% O₂ and 5% CO₂.

The purified α -bungarotoxin was kindly supplied by Dr Michael Raftery of the Department of Chemistry, California Institute of Technology, USA. (*p*Cl-D-Phe⁶,Leu¹⁷)VIP (porcine) and (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide (human) were

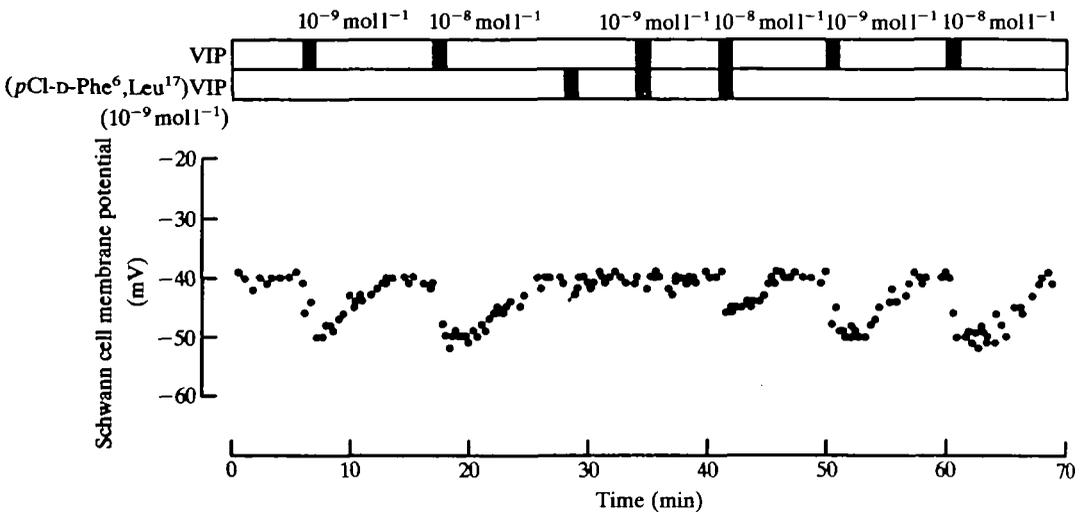


Fig. 1. The blocking actions of (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP on the effects of VIP on the Schwann cell membrane potential. The effects of 1-min pulses of $10^{-9} \text{ mol l}^{-1}$ (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP (hatched bars) are shown on the responses to 1-min pulses of VIP at two different concentrations (filled bar). Each point represents the potential recorded in a different Schwann cell.

obtained from Bachem, UK Ltd. All other drugs were obtained from the Sigma Chemical Co. and included the porcine synthetic form of vasoactive intestinal peptide (VIP).

Results

The effect of VIP blocking agents

VIP can induce a dose-dependent long-lasting hyperpolarization of the membrane potential of the Schwann cell of the squid giant axon (Evans *et al.* 1986). The VIP blocking agent (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP at concentrations of $10^{-8} \text{ mol l}^{-1}$ and above also induced similar long-lasting hyperpolarizations. A 1-min pulse of $10^{-8} \text{ mol l}^{-1}$ (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP produced 50% of the hyperpolarization induced by an equivalent pulse of VIP. However, at a concentration of $10^{-9} \text{ mol l}^{-1}$ (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP did not produce any direct effects on the Schwann cell membrane potential, but could block or substantially reduce the hyperpolarizing effect of 1-min pulses of various concentrations of VIP (Fig. 1). The effects of a 1-min pulse of $10^{-9} \text{ mol l}^{-1}$ VIP were completely blocked, and those due to a 1-min pulse of $10^{-8} \text{ mol l}^{-1}$ VIP were reduced by 50%. Control pulses of 10^{-9} and $10^{-8} \text{ mol l}^{-1}$ VIP applied at the end of the experiment produced hyperpolarizing responses equal to those produced at the beginning of the experiment, indicating the reversible nature of the blockade induced by the blocking agent. The blockade produced by (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP was also dose-

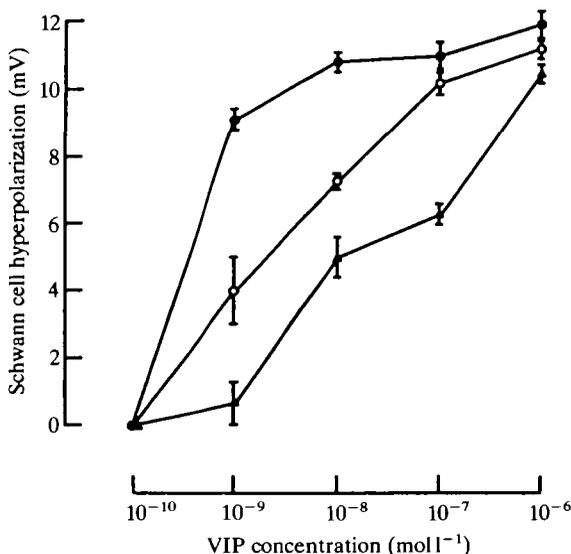


Fig. 2. Dose-response curves for the hyperpolarizing effect of VIP on the Schwann cell membrane potential. Filled circles (●) show control responses. Open circles (○) show the responses to VIP in the presence of $10^{-9} \text{ mol l}^{-1}$ (*N*-Ac-Tyr¹,*D*-Phe²)-GRF(1-29)amide. Filled triangles (▲) show the responses to VIP in the presence of $10^{-9} \text{ mol l}^{-1}$ (*p*Cl-D-Phe⁶,Leu¹⁷)VIP. The values represent the differences \pm s.e. between the membrane potential before VIP application and the maximal response to a 1-min pulse. Each solution was tested on at least four nerve fibres.

dependent since a 1-min pulse of $10^{-10} \text{ mol l}^{-1}$ (*p*Cl-D-Phe⁶,Leu¹⁷)VIP only reduced the hyperpolarization due to a 1-min pulse of $10^{-8} \text{ mol l}^{-1}$ VIP by 15% (not shown).

The second blocking agent, (*N*-Ac-Tyr¹,*D*-Phe²)-GRF(1-29)amide, used in the present study produced a similar direct effect on the Schwann cell membrane potential when applied as a 1-min pulse at concentrations of $10^{-8} \text{ mol l}^{-1}$ and above. It also did not produce any direct effects on membrane potential at a concentration of $10^{-9} \text{ mol l}^{-1}$ (not shown). This blocking agent was also able to block the hyperpolarizing effects of pulses of VIP, but was less effective than the corresponding dose of (*p*Cl-D-Phe⁶,Leu¹⁷)VIP. Fig. 2 shows dose-response curves for the hyperpolarizing effects of 1-min pulses of different concentrations of VIP on the squid Schwann cell membrane potential in the absence, and in the presence, of each of the two blocking agents used at a concentration of $10^{-9} \text{ mol l}^{-1}$. Both blocking agents shifted the dose-response curve to the right and, at all concentrations of VIP tested, (*p*Cl-D-Phe⁶,Leu¹⁷)VIP was a more effective blocking agent than (*N*-Ac-Tyr¹,*D*-Phe²)-GRF(1-29)amide. The apparent K_m for the VIP-induced hyperpolarizations is 230 pmol l^{-1} , and the K_i values for (*p*Cl-D-Phe⁶,Leu¹⁷)VIP and (*N*-Ac-Tyr¹,*D*-Phe²)-GRF(1-29)amide are 16 and 48 pmol l^{-1} , respectively, when calculated from plots of V against V/S . Increasing the concentration of VIP in the test pulses overcame the blocking effects of these two agents in a dose-

dependent manner. At a VIP concentration of $10^{-6} \text{ mol l}^{-1}$ the effects in the presence of the blocking agents were almost identical to those of control pulses of VIP given alone.

Specificity of blocking effects

To test the specificity of the blocking agents for the VIP response, we tested their capacity to block the actions of a range of neurotransmitter substances previously described as being capable of modulating the membrane potential of the Schwann cell of the squid giant axon (see Villegas, 1981, 1984; Reale *et al.* 1986; Evans *et al.* 1986). A 1-min pulse of $10^{-9} \text{ mol l}^{-1}$ (*p*Cl-D-Phe⁶,Leu¹⁷)VIP did not reduce the size of the Schwann cell hyperpolarization induced by 1-min pulses of $10^{-9} \text{ mol l}^{-1}$ substance P, $10^{-7} \text{ mol l}^{-1}$ carbachol or $10^{-6} \text{ mol l}^{-1}$ DL-octopamine, but completely and reversibly blocked the effects of a 1-min pulse of $10^{-9} \text{ mol l}^{-1}$ VIP (Fig. 3). In parallel experiments 1-min pulses of $10^{-9} \text{ mol l}^{-1}$ (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide showed an identical specificity profile (not shown). Thus both blocking agents are specific for the receptors mediating the effects of VIP in this preparation.

Effects of the VIP blocking agents on the endogenous modulation of the Schwann cell

An endogenous modulator is released in the squid giant-axon-Schwann-cell preparation at the time of axonal activation which may be a peptide structurally related to VIP (Evans *et al.* 1986). The effect of this endogenously released modulator on the membrane potential of the Schwann cell could be demonstrated in preparations where all the nicotinic cholinergic responses had been blocked by the application of $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin by stimulating the giant axon at 100 Hz in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine, a dose of octopamine which by itself had no direct effect on the membrane potential (Fig. 4). However, this endogenous component was substantially and reversibly reduced in the presence of both of the blocking agents used in the present study. The pulse of $10^{-9} \text{ mol l}^{-1}$ (*p*Cl-D-Phe⁶,Leu¹⁷)VIP given at the same time as 100 Hz stimulation of the giant axon in the presence of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine was more effective at blocking the endogenous component than a corresponding pulse of (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide (Fig. 4).

Previous experiments suggest that the endogenous activation of those receptors that are activated by exogenously applied VIP plays a role in the production of the normal hyperpolarizing effect induced in the Schwann cell upon axonal stimulation (Evans *et al.* 1986). Fig. 5 shows that 1-min pulses of $10^{-9} \text{ mol l}^{-1}$ of both blocking agents, which had no effect alone, reversibly reduced the hyperpolarizing effect on the Schwann cell of stimulating the giant axon at 100 Hz for 1-min periods. Again (*p*Cl-D-Phe⁶,Leu¹⁷)VIP was more effective than (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide. This result suggests that both the blocking agents used in

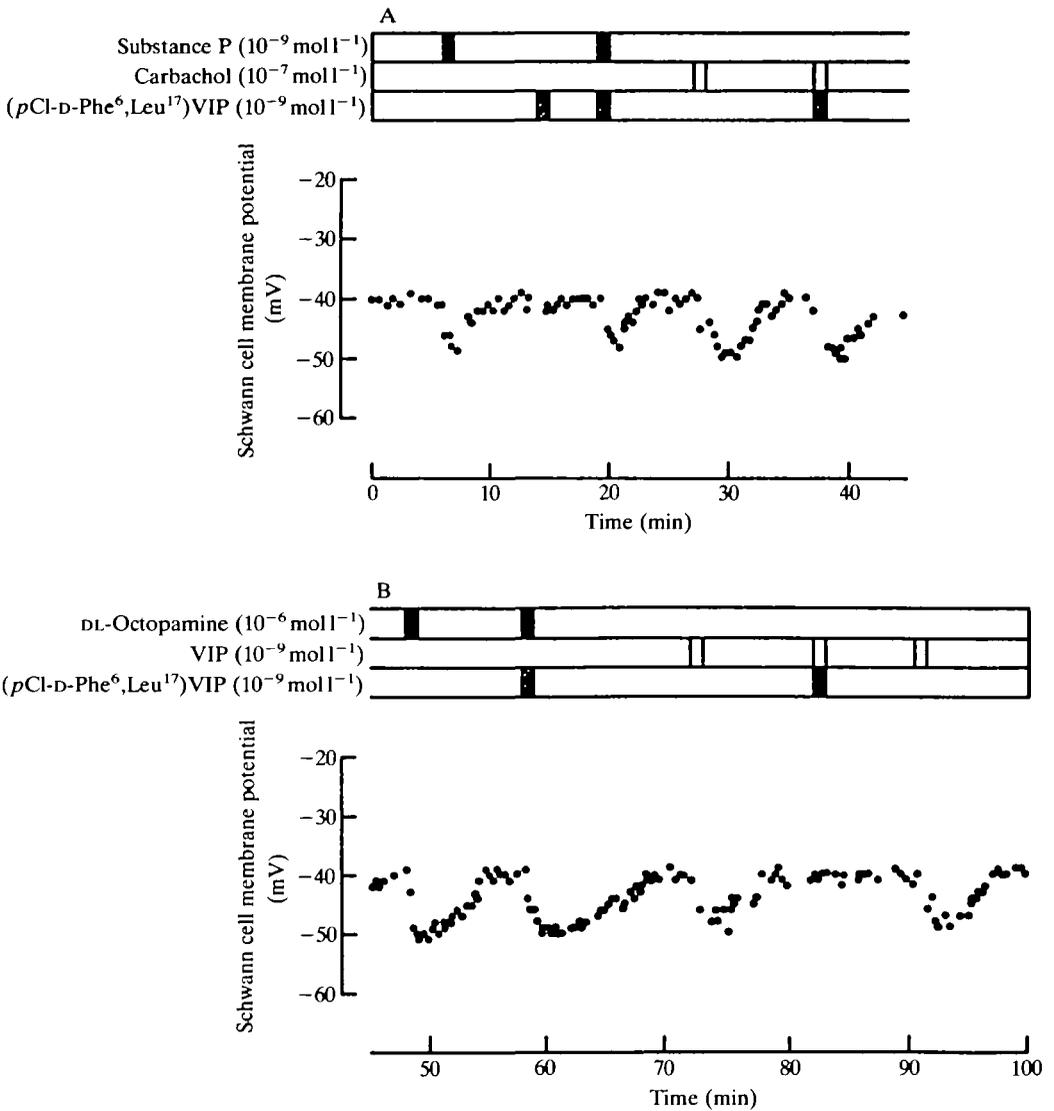


Fig. 3. Specificity of the blocking action of 1-min pulses of $10^{-9} \text{ mol l}^{-1}$ $(p\text{Cl-D-Phe}^6, \text{Leu}^{17})\text{VIP}$ (hatched bars) on the hyperpolarizing effects on the Schwann cell membrane potential of 1-min pulses of (A) $10^{-9} \text{ mol l}^{-1}$ substance P (filled bars) and $10^{-7} \text{ mol l}^{-1}$ carbachol (open bars) and (B) $10^{-6} \text{ mol l}^{-1}$ DL-octopamine (filled bars) and $10^{-9} \text{ mol l}^{-1}$ VIP (open bars). B is a continuation of the experiment shown in A. Each point represents the potential difference recorded in a different Schwann cell.

the present study can reduce the effectiveness of the putative peptidergic potentiation of the cholinergic response which makes up the hyperpolarizing effect of giant axon stimulation under normal circumstances.

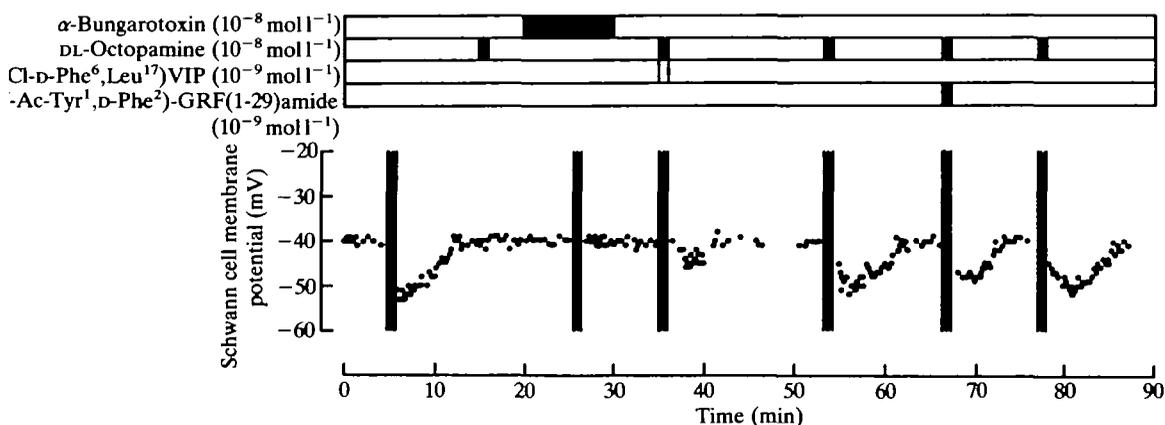


Fig. 4. Actions of antagonists on a non-cholinergic putative peptidergic component of the neurally induced hyperpolarization of the Schwann cell membrane potential. During the intervals indicated by the vertical bars stimuli were delivered to the axon at 100 Hz. The hyperpolarization normally evoked by stimulation of the axon is blocked in the presence of $10^{-8} \text{ mol l}^{-1}$ α -bungarotoxin (hatched bar) (the effects of α -bungarotoxin persist throughout the experiment following its application) but a subthreshold 1-min pulse of $10^{-8} \text{ mol l}^{-1}$ DL-octopamine (filled bars) reveals an additional non-cholinergic hyperpolarizing component when applied at the same time as the 100 Hz stimulation. This non-cholinergic component is reduced in the presence of 1-min pulses of $10^{-9} \text{ mol l}^{-1}$ (pCl-D-Phe⁶,Leu¹⁷)VIP (open bar) and of $10^{-9} \text{ mol l}^{-1}$ (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide (stippled bar). Each point represents the potential difference recorded in a different Schwann cell.

Discussion

The receptors mediating the hyperpolarizing action of exogenously applied VIP on the Schwann cells surrounding the squid giant axon are blocked in a dose-dependent manner by both (pCl-D-Phe⁶,Leu¹⁷)VIP and (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide, with the former being more effective than the latter. The effects of both blockers are reversible and both can be shown by kinetic analysis to be competitive antagonists of the VIP-induced responses. Both antagonists show weak agonist activity at concentrations of $10^{-8} \text{ mol l}^{-1}$ and above. A 1-min pulse of either compound at $10^{-8} \text{ mol l}^{-1}$ shows 50% of the hyperpolarizing effect on the Schwann cell membrane potential of that produced by an equivalent pulse of VIP.

Growth hormone-releasing factor (GRF) analogues were originally examined for their antagonistic actions on VIP receptors since GRF has a considerable sequence homology with the family of peptides that includes VIP, peptide histidine isoleucine, secretin, glucagon and gastric inhibitory peptide (Spiess *et al.* 1983; Spiess *et al.* 1982). (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide was shown to inhibit selectively adenylate cyclase activity stimulated by VIP, but not that stimulated by secretin in rat pancreatic plasma membranes, and also to inhibit [¹²⁵I]iodo-VIP binding in the same preparation (Waelbroeck *et al.* 1985). This antagonist produced a competitive inhibition of the VIP receptors in the rat

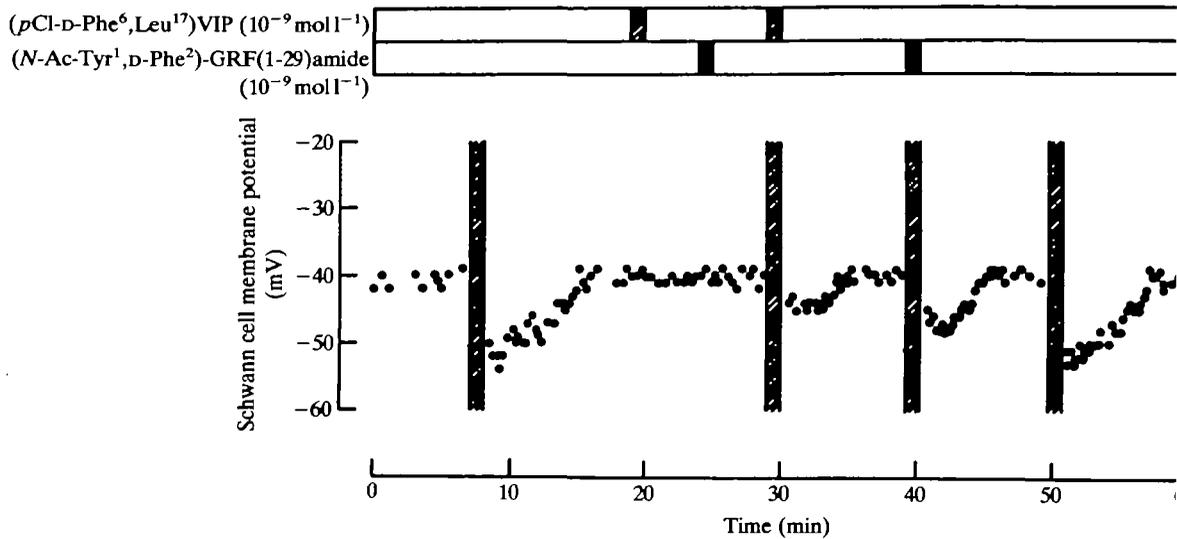


Fig. 5. The blocking action of the antagonists on the neurally induced hyperpolarization of the Schwann cell membrane potential. During the intervals indicated by the vertical bars stimuli were delivered to the axon at 100 Hz. 1-min pulses of 10^{-9} mol l^{-1} (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP (hatched bars) and 10^{-9} mol l^{-1} (*N-Ac-Tyr*¹,*D-Phe*²)-GRF(1-29)amide (filled bars), which have no direct effect on their own, reduce the response to 100 Hz stimulation indicating that the latter effect normally represents a cholinergic component which is potentiated by the release of an endogenous VIP-like peptide.

pancreatic plasma membranes in a fashion similar to that described in the present paper for the receptors activated by VIP on the membrane of the squid Schwann cell. In contrast to the results obtained in the present study, (*N-Ac-Tyr*¹,*D-Phe*²)-GRF(1-29)amide did not show any weak agonist activity in the rat pancreatic plasma membrane preparation. VIP has recently been shown to elicit a potassium current in follicle-enclosed *Xenopus* oocytes by a cyclic-AMP-mediated process (Woodward & Miledi, 1987; Reale *et al.* 1987), but the receptor mediating these effects does not appear to be blocked by (*N-Ac-Tyr*¹,*D-Phe*²)-GRF(1-29)amide at concentrations up to $1.5 \mu\text{mol} l^{-1}$ (Woodward & Miledi, 1987). This suggests that (*N-Ac-Tyr*¹,*D-Phe*²)-GRF(1-29)amide may be able to distinguish between different subclasses of receptor for VIP both of which mediate their actions *via* an activation of adenylate cyclase activity.

(*pCl-D-Phe*⁶,*Leu*¹⁷)VIP has been shown to be a selective competitive antagonist of VIP receptors in a number of biological systems that also contain receptors for the related peptides secretin, glucagon and GRF (Pandol *et al.* 1986). These include VIP-stimulated amylase release from the exocrine pancreas and VIP-stimulated short-circuit current changes in a colonic tumour cell line. In the exocrine pancreas assay (*pCl-D-Phe*⁶,*Leu*¹⁷)VIP acts as a weak agonist at higher concentrations in a fashion similar to that described in the present paper for its actions on the membrane potential of the squid Schwann cell.

In the present study both antagonists were shown selectively to antagonize the action of the receptors mediating the hyperpolarizing actions of VIP on the membrane potential of the Schwann cell of the squid giant axon. At a concentration of 10^{-9} mol l⁻¹ neither antagonist shows any agonist activity and neither antagonist blocks the receptors mediating the hyperpolarizing actions in this preparation of acetylcholine (Villegas, 1975), octopamine (Reale *et al.* 1986) or substance P (Evans *et al.* 1986). Thus the two antagonists used in the present study show the same antagonistic selectivity profile in the squid preparation as that for APP (Evans *et al.* 1986) which has been shown to block some of the actions of VIP in other preparations (Lundberg, 1981; Fredholm & Lundberg, 1982; Karpinski *et al.* 1984).

An endogenous non-cholinergic modulator has been suggested to be released in the squid giant-axon-Schwann-cell preparation at the time of axonal activation which may be a peptide structurally related to VIP (Evans *et al.* 1986). The presence of this modulator was first demonstrated in preparations where the nicotinic cholinergic receptors were blocked by α -bungarotoxin and was revealed as a hyperpolarization of the Schwann cell membrane potential in response to axonal activation only in the presence of low potentiating concentrations of DL-octopamine which had no direct effects on their own. This suggests that this endogenous component does not normally produce a direct effect on the Schwann cell membrane potential. Rather, its role is to potentiate the magnitude of the Schwann cell membrane response to the cholinergic signal released from the Schwann cell itself in response to stimulation of the giant axon. This non-cholinergic component has previously been shown to be blocked by APP (Evans *et al.* 1986) but the mechanism of action of this APP-mediated inhibition is not known and, in addition, doubts have been expressed about the reproducibility of the actions of APP in blocking VIP effects in other preparations (see Karpinski *et al.* 1984). In the present study we have demonstrated that this non-cholinergic endogenous component is also blocked by both the antagonists used in experiments where its presence is demonstrated by the potentiating action of octopamine. Further, both the antagonists also reduce the size of the hyperpolarization of the Schwann cell induced by firing the giant axon in the absence of α -bungarotoxin, indicating that they can also reduce the degree of potentiation of the cholinergic response produced by the release of the endogenous modulator. In both these sets of experiments (*p*Cl-D-Phe⁶,Leu¹⁷)VIP is a more potent blocking agent than (*N*-Ac-Tyr¹,D-Phe²)-GRF(1-29)amide. This parallels their blocking potency on the hyperpolarizing effects on the Schwann cell membrane potential produced by the exogenous application of VIP.

Thus the use of the two selective VIP antagonists has strengthened the case for the existence of VIP receptors on the membrane of the Schwann cell of the squid giant axon with pharmacological similarities to VIP receptors described in a number of vertebrate preparations. In addition, it enhances the idea that an endogenous VIP-like peptide is released in the squid giant-axon-Schwann-cell preparation upon stimulation of the giant axon to potentiate the nicotinic

cholinergic response of the Schwann cells. Experiments are currently in progress to determine if this putative VIP-like peptide is co-released from the Schwann cells along with acetylcholine or if it is released from the giant axon upon stimulation.

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