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SOMATOSTATIN-MEDIATED INHIBITORY POSTSYNAPTIC POTENTIAL IN SYMPATHETICALLY DENERVATED GUINEA-PIG SUBMUCOSAL NEURONES

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SUMMARY

1. Intracellular recordings were made from submucosal neurones in guinea-pig ileum. In some animals, the extrinsic (sympathetic) nerves to the submucosal plexus were severed 5–7 days previously. The actions of somatostatin and somatostatin analogues on membrane potential, membrane current and inhibitory postsynaptic potentials (IPSPs) were examined.

2. Somatostatin, somatostatin(1–28), [D-Trp^8]somatostatin and the somatostatin analogue CGP 23996 all produced equivalent maximum hyperpolarizations or outward currents; half-maximal concentrations (EC_{50} values) were 9–11 nM. The somatostatin analogue MK 678 had an EC_{50} of 0·9 nM. Extrinsic sympathectomy did not alter concentration–response relations for somatostatin or its analogues.

3. Somatostatin (> 100 nM) produced hyperpolarization or outward current that declined almost completely during superfusion for 2–4 min; decline of the somatostatin current was exponential with a time constant of 30 s in the presence of 2 μM somatostatin. Desensitization was not altered by extrinsic denervation.

4. Recovery from desensitization was rapid and followed the time course of agonist wash-out. Forskolin, phorbol esters, dithiothreitol, hydrogen peroxide, concanavalin A, or reducing temperature from 35 to 29 °C did not alter the time course, degree of, or recovery from desensitization.

5. The somatostatin-induced desensitization was of the homologous type; no cross-desensitization to opiate or α_2-adrenoceptor agonists (which activate the same potassium conductance) occurred.

6. Somatostatin desensitization did not alter the adrenergic IPSP seen in sympathetically innervated preparations but abolished the non-adrenergic IPSP recorded from normal preparations and from preparations in which the extrinsic sympathetic nerve supply had been surgically removed.

7. The selective blockade of the non-adrenergic IPSP by the homologous-type somatostatin desensitization characterized in the present study provides strong support for the hypothesis that somatostatin is the neurotransmitter underlying the non-adrenergic IPSP in both normal and extrinsically denervated submucosal neurones.

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INTRODUCTION

Somatostatin is contained in, and released from, many central and peripheral neurones and therefore has been implicated as a transmitter in the nervous system (Vale, Rivier & Brown, 1977; Iversen, Iversen, Bloom, Douglas, Brown & Vale, 1978). The most common neuronal action of somatostatin is one of inhibition with its most common cellular mechanism of action being a membrane hyperpolarization brought about by activation of inwardly rectifying potassium channels (Mihara, North & Surprenant, 1987 *a*; Inoue, Nakajima & Nakajima, 1988; Lewis & Clapham, 1989; Sims, Lussier & Kraicer, 1991) . This cellular mechanism underlies inhibitory postsynaptic potentials (IPSPs) at several muscarinic, adrenergic and γ-amino-butyric acid ($GABA_B$) synapses (Hartzell, Kuffler, Stickgold & Yoshikami, 1977; Dodd & Horn, 1983; Surprenant & North, 1988; Mihara & Nishi, 1989; Nicoll, Malenka & Kauer, 1990); somatostatin might also be expected to mediate similar IPSPs at sites where it is known to be released.

Currently the case for a somatostatin-mediated postsynaptic potential is most developed for guinea-pig submucosal neurones (see Surprenant, 1989). Somatostatin is contained in nerve terminals surrounding submucosal neurones, the source of which is twofold: somatostatin is co-localized with noradrenaline in only those sympathetic neurones which innervate submucosal neurones, and somatostatin is also co-localized in a subpopulation of cholinergic neurones in the myenteric plexus which innervate submucosal neurones (Costa, Furness, Llewellyn-Smith, Davies & Oliver, 1980; Costa & Furness, 1984; Furness & Costa, 1987). Here, a non-adrenergic, non-cholinergic IPSP can be recorded after blockade of the sympathetically mediated IPSP with α_2-adrenoceptor antagonists. This non-adrenergic IPSP appears to become especially prominent a few days after surgical removal of the extrinsic sympathetic nerves, but it is not present after concomitant removal of the myenteric plexus and sympathetic nerves, a procedure which results in the disappearance of all somatostatin-containing nerve terminals surrounding submucosal neurones (Mihara, Nishi, North & Surprenant, 1987 *b*; Bornstein, Costa & Furness, 1988). Both adrenergic and non-adrenergic IPSPs result from the same ionic mechanism, an activation of a pertussis toxin-sensitive inwardly rectifying potassium conductance (Mihara *et al.* 1987 *b*; Surprenant & North, 1988). Furthermore, exogenous somatostatin and opiates mimic the effects of noradrenaline: they not only hyperpolarize the same cells, but they open the same channels in excised membrane patches (Mihara *et al.* 1987 *a*, *b*; Surprenant & North, 1988; Shen, North & Surprenant, 1992). Antagonists of opioid receptors do not affect the non-adrenergic IPSP (Mihara *et al.* 1987 *b*; Bornstein, Costa & Furness, 1988), and this implies that somatostatin may be the transmitter. However, a final criterion to support a role for somatostatin as mediator of the non-adrenergic IPSP has yet to be met: selective antagonism of this IPSP has not been described previously.

The main aims of the present study were to characterize and compare in more detail the properties of the postsynaptic somatostatin response and the IPSPs in submucosal neurones from normal and extrinsically denervated preparations in an attempt to further elucidate the possible role of somatostatin as the neurotransmitter underlying the non-adrenergic IPSP.

METHODS

Preparations of submucosal plexus were obtained from the small intestine of young adult guinea-pigs (200–400 g); animals were killed by cervical dislocation and carotid exsanguination (method approved by the Animal Care and Use Committee of Oregon Health Sciences University). Methods of dissection and focal external stimulation have been described previously (Mihara *et al.* 1987*a*,*b*). In some experiments, a larger artery in the mesenteric arcade (outside diameter, 0·8–1·3 mm) was cannulated prior to opening the intestine and the vasculature gently perfused with physiological saline solution containing very dilute blue food dye, thus allowing visualization of the area fed by the cannulated mesenteric branch. The intestine was then opened and care taken to dissect this region of the submucosal plexus leaving the cannulated external mesenteric vessel attached, after which the cannula was removed and replaced by a suction electrode on the external blood vessel; this electrode was used for selective stimulation of the extrinsic nerve fibres (see diagram in Fig. 6). Physiological saline solution of the following composition (mM) flowed continuously through the organ bath (0·5 ml volume): NaCl, 126; NaH_2PO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 2·5; KCl, 5; $NaHCO_3$, 25; glucose, 11; gassed with 95 % O_2 and 5 % CO_2; the temperature was maintained at 34–36 °C.

Intracellular microelectrodes (55–80 MΩ) were used for voltage and current recordings via an Axoclamp 2A single-electrode voltage clamp amplifier. All methods of recording and drug applications by superfusion, ionophoresis and pressure-ejection have been described in detail previously (Mihara *et al.* 1987*a*,*b*; Surprenant & North, 1988). Extrinsic denervations were performed according to the method of Furness & Costa (Furness & Costa, 1978; Costa & Furness, 1984; Bornstein *et al.* 1988). Only those preparations in which no catecholamine fluorescence was visible using the glyoxylic acid (Furness & Costa, 1975) or formaldehyde–glutaraldehyde method (Furness, Costa & Wilson, 1978) were included in the data for extrisically denervated preparations.

The amplitude times the half-duration of the IPSP was measured in all experiments and these values were used to construct concentration–response curves for experiments examining actions of antagonists (e.g. Fig. 7).

Drugs used were: somatostatin, somatostatin(1–14), somatostatin(1–28), [D-Trp^8]somatostatin, [Met^5]enkephalin, tetrodotoxin and noradrenaline (Sigma, St Louis, MO, USA); phentolamine hydrochloride (Ciba-Geigy, Basel, Switzerland); idazoxan (gift from Reckitt & Colman, Hull, Yorkshire, UK); (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304, gift from Pfizer, New York, NY, USA). Professor T. Reisine (University of Pennsylvania) generously provided the somatostatin analogues cyclo(*N*-Me-Ala-Tyr-D-Trp-Lys-Phe) (MK 678, originally synthesized by Ciba-Geigy) and des-Ala^1,Gly^2-[desamino-Cys^3,Tyr^{11}]-3,14-dicarbasomatostatin (CGP 23996, originally synthesized by Merck, West Point, PA, USA).

RESULTS

Somatostatin actions and desensitization

Sympathetic innervation intact

Somatostatin, somatostatin(1–28), [D-Trp^8]somatostatin, CGP 23996 and MK 678 all caused a concentration-dependent membrane hyperpolarization or outward current at the resting potential (Fig. 1). Each agonist produced the same maximum response ($\pm$ 5 %) in a given neurone; maximum hyperpolarization from a membrane potential of −55 mV was 26 $\pm$ 2 mV ($n = 43$) and maximum outward current at the holding potential of −60 mV was 558 $\pm$ 32 pA ($n = 22$). MK 678 was approximately 10-fold more potent than any of the other agonists; the half-maximal concentration (EC_{50}) for MK 678 was 0·9 nM and for the other agonists it was 9–11 nM (Fig. 1*B*). Somatostatin(1–14) was without effect on submucosal neurones ($n = 3$, Fig. 1*B*).

Superfusion for 1–5 min with any of these agonists at concentrations from 0·1 to 50 nM produced maintained responses (Fig. 1*A*); higher concentrations resulted in marked tachyphylaxis (Fig. 2). The average time course of decay of the outward

current in the continued presence of somatostatin is summarized in Fig. 3; the decay was well fitted to a single exponential function with time constants (τ) of 83 ± 2 s ($n = 10$) and 29 ± 2 s ($n = 8$) in the presence of 200 nM and 2 μM somatostatin respectively. Results from similar experiments in which noradrenaline, which opens

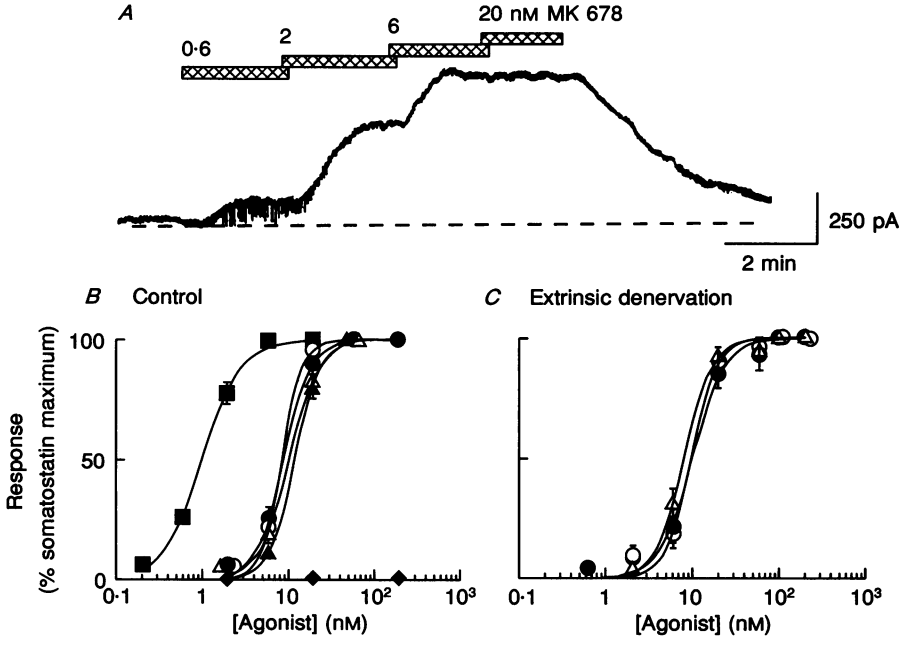

Fig. 1. Concentration–response relations for somatostatin and somatostatin analogues to increase potassium conductance in submucosal neurones. *A*, outward current produced in response to superfusion with increasing concentrations of the somatostatin receptor agonist, MK 678; agonist was applied for durations indicated by shaded bars. Holding potential, −60 mV. Outward current was maintained for periods of up to 10 min during superfusion with these concentrations of agonist. *B*, concentration–response curves for all agonists examined in normal preparations: somatostatin (●), MK 678 (■), CGP 23996 (▲), [D-Trp^8]somatostatin (○), somatostatin(1–28) (△) and somatostatin(1–14) (◆). Each point is mean ± S.E.M. from 6–20 cells, except for somatostatin(1–14) where $n = 3$ for each point. *C*, concentration–response curve for agonists examined in extrinsically denervated preparations; somatostatin (●), somatostatin(1–28) (△) [D-Trp^8]somatostatin (○). Each point is mean ± S.E.M. from 4–6 cells.

the same set of potassium channels as does somatostatin (Mihara *et al.* 1987*a*; Surprenant & North, 1988; Shen *et al.* 1992), was used as an agonist are also shown for comparison; the outward current did not decline even when noradrenaline was 300 times its EC_{50} concentration (Fig. 3).

Neither [Met^5]enkephalin nor α_2-adrenoceptor agonists showed cross-desensitization when applied in the continued presence of somatostatin at a concentration which produced maximum tachyphylaxis. An example is shown in Fig. 4*A*; the α_2-adrenoceptor agonist UK 14304 (at 10 times its EC_{50}) produced a maintained outward current of 320 pA; a similar outward current of 320 pA was recorded when

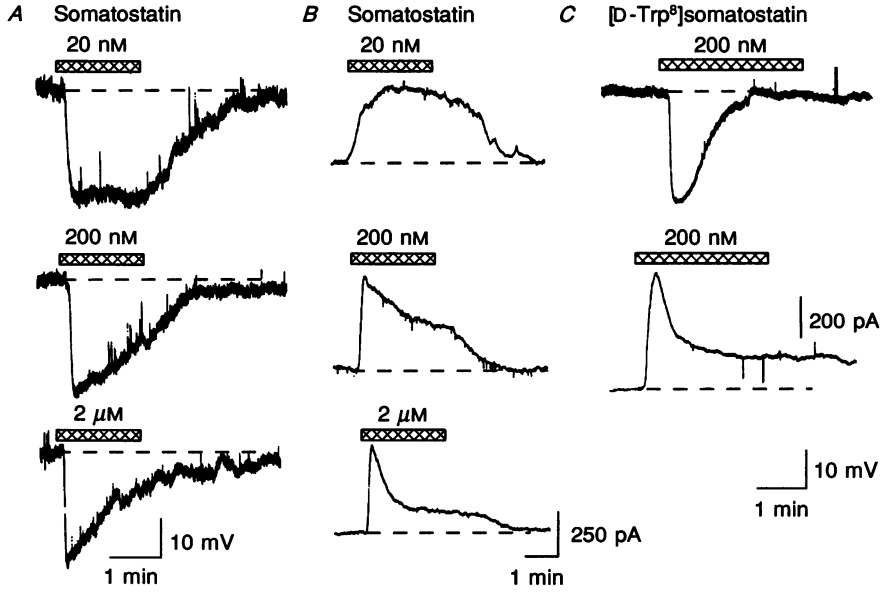

Fig. 2. Desensitization of somatostatin-induced hyperpolarization (*A*) and outward current (*B*). *A*, membrane potential recorded from one neurone during superfusion for 2 min with 20, 200 and 2000 nM somatostatin (as indicated); the higher concentrations of somatostatin produced a hyperpolarization which faded in the continued presence of agonist. Membrane potential, −52 mV. *B*, outward current at holding potential of −60 mV recorded from another neurone in response to superfusion with 20, 200 and 2000 nM somatostatin. *C*, hyperpolarization (upper trace) and outward current (lower trace) recorded from one neurone in response to superfusion with 200 nM [D-Trp^8]somatostatin; this concentration caused rapid tachyphylaxis during application; resting and holding potential, −55 mV.

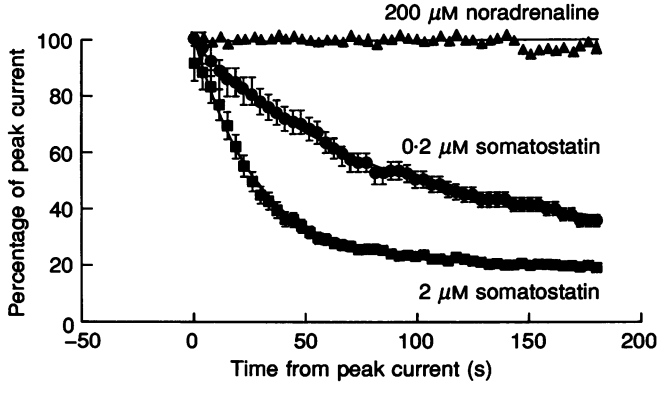

Fig. 3. Summary of time course of desensitization of outward current produced by 0·2 μM (●) and 2 μM (■) somatostatin. Results are expressed as percentage of peak current from experiments as shown in Fig. 2*B* during which 3–4 min superfusions with somatostatin were carried out; each point is mean ± S.E.M. of 4 experiments. Lines drawn through points are exponential fits. Also shown are results from similar experiments in which noradrenaline (200 μM which is 200 times the EC_{50} concentration) was applied; each point is mean ± S.E.M. of 3 experiments with error bars within limits of symbol.

UK 14304 was re-applied in the presence of somatostatin (2 μM) but after the response to somatostatin had faded to 15% of its peak. Similar results were observed with noradrenaline (20 μM; $n = 8$) and [Met^5]enkephalin (20 μM; $n = 6$)

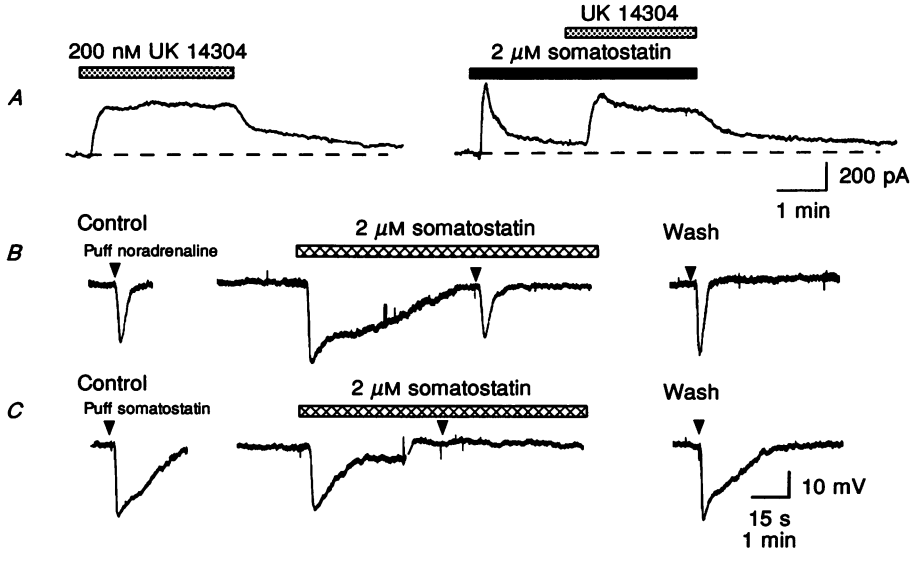

Fig. 4. Desensitization by somatostatin does not cause cross-desensitization by α_2-adrenoceptor agonists. *A*, outward current recorded from one neurone during superfusion with UK 14304 (left); no tachyphylaxis of response occurred during the 3 min superfusion period. A similar amplitude outward current was produced by the same concentration of UK 14304 when it was applied during the continued presence of a desensitizing concentration of somatostatin (right). Holding potential, −60 mV. *B* and *C*, the hyperpolarization produced by brief (5 ms) pressure ejection of noradrenaline was unaltered during desensitization of the somatostatin-induced hyperpolarization (*B*) but the hyperpolarization to pressure ejection of somatostatin was abolished during the desensitization produced by superfusion with somatostatin (*C*). The faster time calibration in *B* and *C* refers to recordings of pressure-ejection responses; slower time calibration refers to recording during superfusion of somatostatin.

superfusion in the absence and presence of somatostatin, and with brief applications of noradrenaline by pressure pulse application ($n = 7$; Fig. 4*B*). The hyperpolarization caused by brief applications of somatostatin were abolished during somatostatin desensitization ($n = 5$; Fig. 4*C*).

The outward current evoked by somatostatin recovered quickly from desensitization. Brief pressure-ejection pulses (5 ms) of somatostatin were applied at approximately 2 min intervals before, during and after superfusion with somatostatin (2 μM), or in control experiments in which noradrenaline (20 μM) was applied by superfusion. The outward current to noradrenaline does not fade (e.g. Fig. 3) but the somatostatin response is occluded during application of noradrenaline because they open common channels (Surprenant & North, 1988). Thus, we were able to compare the time course of recovery of sensitivity to somatostatin after somatostatin desensitization (●, Fig. 5) with the time course of recovery of

sensitivity to somatostatin after noradrenaline wash-out (○, Fig. 5). Recovery of the somatostatin response occurred with the same time course as noradrenaline wash-out ($n = 7$; Fig. 5).

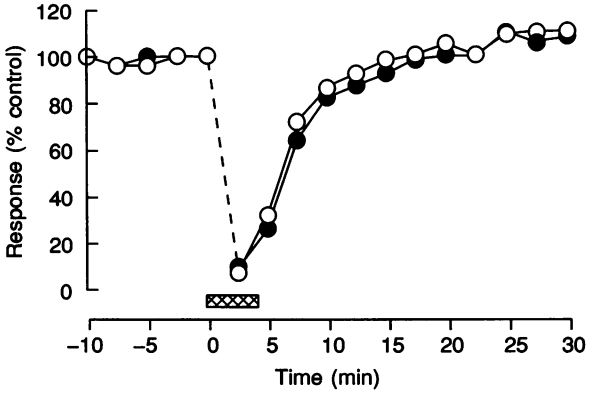

Fig. 5. Time course of recovery from desensitization by somatostatin. Each point represents the amplitude of outward current in response to pressure-ejection of somatostatin (as percentage of response at −10 min). ●, somatostatin (2 μM) was superfused for 4 min at time zero (indicated by bar); this produced an outward current which faded to 12 % of peak current by 3 min. ○, noradrenaline (200 μM) was superfused for 4 min at time zero; this produced an outward current which was maintained (± 5 %) for the duration of application. Data are from recordings obtained in one neurone.

We examined the effects of a number of substances which are known to alter properties of desensitization in other receptor systems, such as the β-adrenoceptor (Lefkowitz & Caron, 1987; Sibley, Benovic, Caron & Lefkowitz, 1988). We measured three parameters: (1) the time course of tachyphylaxis to somatostatin, (2) the concentration of somatostatin producing tachyphylaxis and (3) the degree of tachyphylaxis produced by 2 μM somatostatin (as a percentage of peak current remaining at 3 min in somatostatin). The following substances produced no significant effect on any of these three parameters (Student's t test, $P > 0{\cdot}2$): forskolin (2 μM, $n = 12$); phorbol 12,13-dibutyrate (2 μM, $n = 5$), dithiothreitol (4 mM, $n = 10$), H_2O_2 (100 nM, $n = 6$), concanavalin A (200 μg/ml, $n = 4$), the δ-opiate receptor agonist, [D-Pen^{2,5}]enkephalin (20 μM, $n = 5$), and the α_2-adrenoceptor partial agonist, clonidine (2 μM, $n = 4$). Dithiothreitol and hydrogen peroxide decreased the peak somatostatin response by 45–80 %. Decreasing temperature from 35 to 29 °C also did not alter the properties of the tachyphylaxis in response to somatostatin ($n = 3$).

Sympathetic denervation

Concentration–response relations for outward currents to somatostatin, [D-Trp^8]somatostatin and somatostatin(1–28) were unaltered in preparations from which the sympathetic nerves had been removed 5–8 days previously (Fig. 1*C*). The properties of desensitization of outward current or hyperpolarization to somatostatin were also unaffected by extrinsic sympathetic denervation ($n = 4$).

The adrenergic IPSP

Sympathetic innervation intact

Focal stimulation of interganglionic connectives is well known to evoke an α_2-adrenergic IPSP in submucosal neurones which is due to the release of

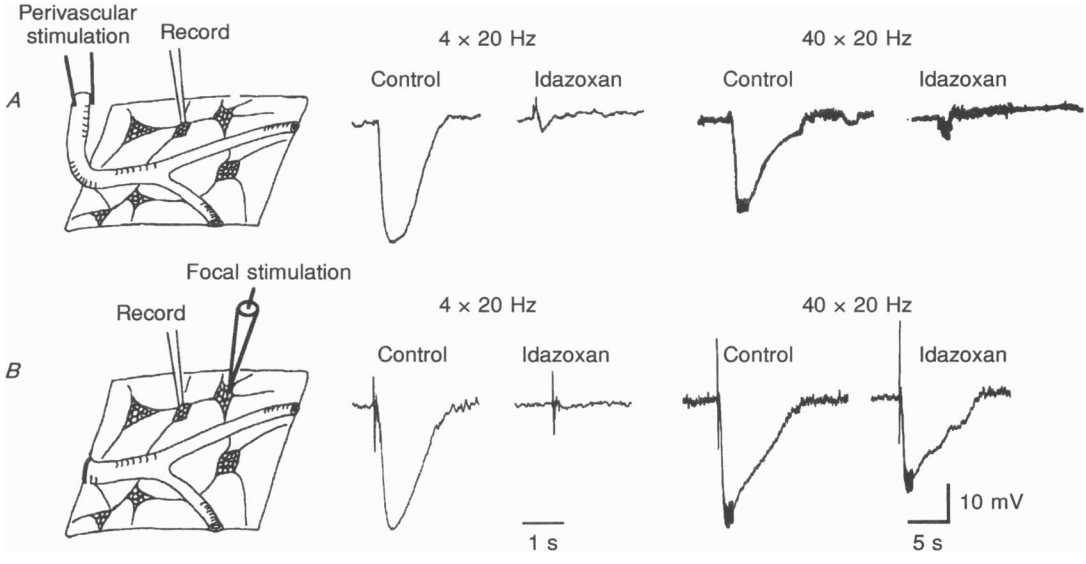

Fig. 6. *A*, adrenergic IPSP evoked by selective stimulation of the extrinsic nerve supply. The larger mesenteric artery feeding the submucosal vasculature was used for stimulation; stimulation at 20 Hz for 4 pulses or 40 pulses (as indicated) evoked IPSPs which were abolished by idazoxan (2 μM). Note absence of fast nicotinic EPSPs; no nicotinic receptor blockers present. *B*, adrenergic and non-adrenergic IPSP evoked by focal stimulation of interganglionic connectives. Idazoxan (2 μM) abolished the IPSP in response to 4 stimuli at 20 Hz but inhibited the IPSP in response to 40 stimuli by about 40 %. Hexamethonium (20 μM) present to inhibit fast nicotinic EPSPs. Note IPSPs in response to brief stimuli are shown on fast time scale, those in response to prolonged stimulation on slower time scale.

noradrenaline from sympathetic nerves (North & Surprenant, 1985; Mihara, Katayama & Nishi, 1985; see Surprenant, 1989), but it is not clear whether a non-adrenergic inhibitory transmitter may be co-released from these extrinsic nerves. There is also some question as to the concentrations of α_2-adrenoceptor antagonists which completely suppress the adrenergic IPSP (e.g. North & Surprenant, 1985; Bornstein *et al.* 1988). Therefore, we compared the α_2-adrenoceptor inhibition of the IPSP to brief or prolonged stimuli (4 or 40 pulses at 20 Hz) evoked by selective stimulation of the extrinsic nerves or by focal stimulation of interganglionic connectives (shown diagrammatically in Fig. 6).

Perivascular stimulation of the extrinsic nerve supply evoked IPSPs without preceding fast EPSPs (Fig. 6*A*). Guanethidine (10 μM) abolished IPSPs elicited by brief or long trains of perivascular stimuli ($n = 4$). Perivascular stimulation often elicited slow EPSPs following the IPSP. These slow EPSPs were not examined in

any detail in the present study; however, it was noted that they were not inhibited by guanethidine and were not observed after extrinsic denervation. Therefore, we assume their origin is most probably from extrinsic sensory fibres. The α_2-adrenoceptor antagonist, idazoxan, abolished IPSPs evoked by brief (4 pulses at

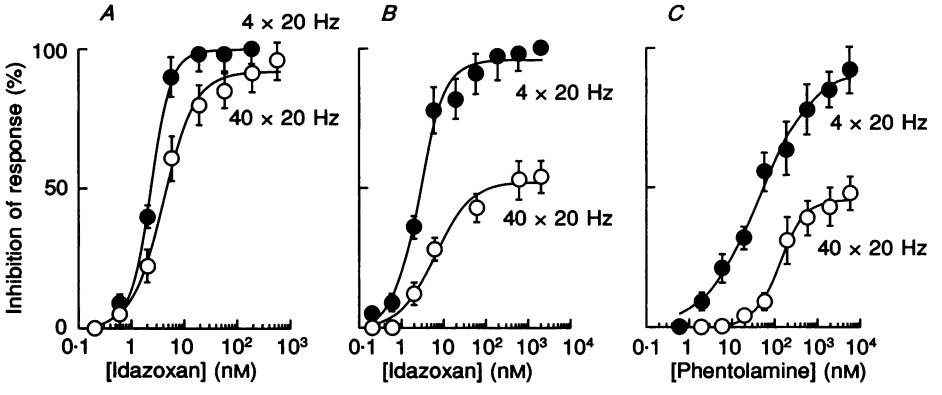

Fig. 7. Summary of inhibition of IPSPs evoked by perivascular stimulation (*A*) or by focal interganglionic stimulation (*B* and *C*) by idazoxan (*A* and *B*) and phentolamine (*C*). ●, IPSPs in response to brief stimuli; ○, IPSPs in response to prolonged stimuli. Each point is mean ± S.E.M. of 3–9 experiments.

20 Hz) stimuli delivered by either extrinsic perivascular (Fig. 6*A*) or interganglionic stimulating electrodes (Fig. 6*B*); half-maximal inhibition (IC_{50} concentration) occurred at 3–5 nM with either stimulating method (Fig. 7*A* and *B*). Idazoxan also abolished IPSPs evoked by prolonged stimulation using extrinsic perivascular electrodes (IC_{50} = 4 nM) but inhibited IPSPs evoked by prolonged stimulation with interganglionic electrodes by a maximum of 50 % (Figs 6 and 7). Phentolamine inhibited IPSPs in response to four and forty stimuli by a maximum of 92 and 48 % respectively when interganglionic stimulating electrodes were used (Fig. 7*C*); the IC_{50} for inhibition of IPSP in response to four stimuli was 55 nM. Phentolamine (0·2 and 2 μM) inhibited IPSPs in response to extrinsic perivascular stimulation with forty pulses by 65 ± 6 and 88 ± 8 % (n = 5); other concentrations were not examined.

Extrinsic denervation

Perivascular stimulation of extrinsically denervated preparations failed to elicit any response (n = 6), even in neurones in which interganglionic stimulation evoked non-adrenergic IPSPs (n = 2, see below).

The non-adrenergic IPSP

Sympathetic innervation intact

Inhibitory synaptic potentials of amplitudes > 3 mV were never recorded in the presence of idazoxan (2 μM) in response to brief stimuli (1–4 pulses at 20 Hz, n > 25; Fig. 8*A*). IPSPs recorded in response to prolonged stimulation (10–100 pulses at 20

or 30 Hz) in the presence of adrenoceptor blockers in normal preparations have been described previously (Mihara *et al.* 1987 *b*; Bornstein *et al.* 1988) and are shown in Figs 6 and 9.

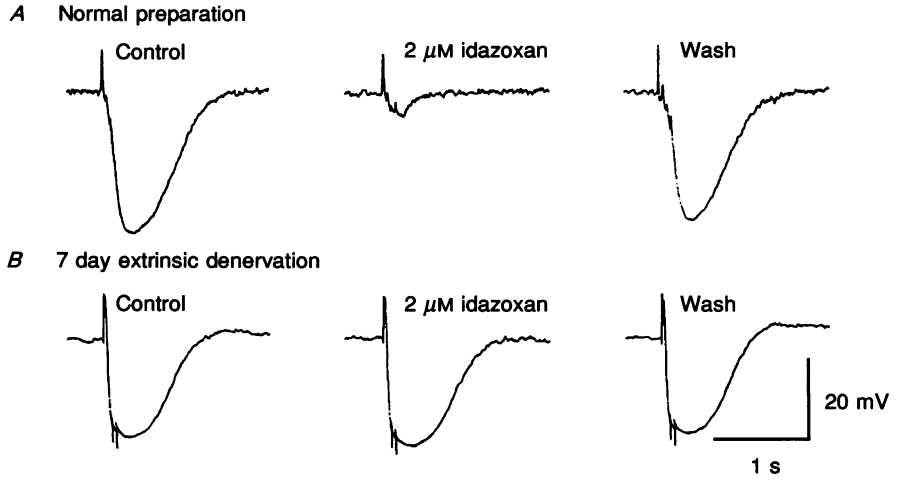

Fig. 8. Idazoxan-sensitive IPSP evoked by 3 stimuli at 20 Hz in a normal preparation (*A*) and idazoxan-insensitive IPSP evoked by similar stimulus in a preparation in which extrinsic nerves had been removed 7 days prior to experiment. Focal interganglionic stimulation used in each case. Note minimal differences in amplitude and time course between adrenergic (*A*) and non-adrenergic (*B*) IPSPs. Hexamethonium (30 μM) present in order to inhibit nicotinic fast EPSPs.

Extrinsic denervation

A non-adrenergic IPSP could be recorded in response to the short train (4 pulses at 20 Hz) of interganglionic stimuli in preparations in which extrinsic denervation had been performed 5–7 days previously (see also Bornstein *et al.* 1988). This non-adrenergic IPSP was unaltered by a concentration of idazoxan (2 μM) which completely abolished the IPSP in response to the same stimulus in normally innervated preparations, and this non-adrenergic IPSP showed remarkably few differences in amplitude or time course to the adrenergic IPSP recorded in normal preparations (Fig. 8). The average amplitude and half-duration of the adrenergic IPSP recorded in normal solution in response to four pulses from −50 mV was 29 ± 2·3 mV and 763 ± 44 ms ($n = 12$); these values were 23 ± 3 mV and 750 ± 58 ms ($n = 10$) for the similarly evoked non-adrenergic IPSP in extrinsically denervated preparations and were not significantly different ($P > 0{\cdot}1$). As reported previously (Mihara *et al.* 1987 *b*; Borstein *et al.* 1988), the non-adrenergic IPSP was unaltered by muscarinic receptor antagonists (2 μM 4-DAMP, $n = 7$), naloxone (2 μM, $n = 7$) or other adrenoceptor blockers (phentolamine or phenoxybenzamine, 2 μM, $n = 5$). Dopamine receptor antagonists, SCH 23390 and sulpiride (1 μM, $n = 4$ for each), and 5-HT receptor antagonists, spiperone, ketanserin and ondansetron (1 μM, $n = 2$ for each), did not inhibit the non-adrenergic IPSP. The non-adrenergic IPSP was also

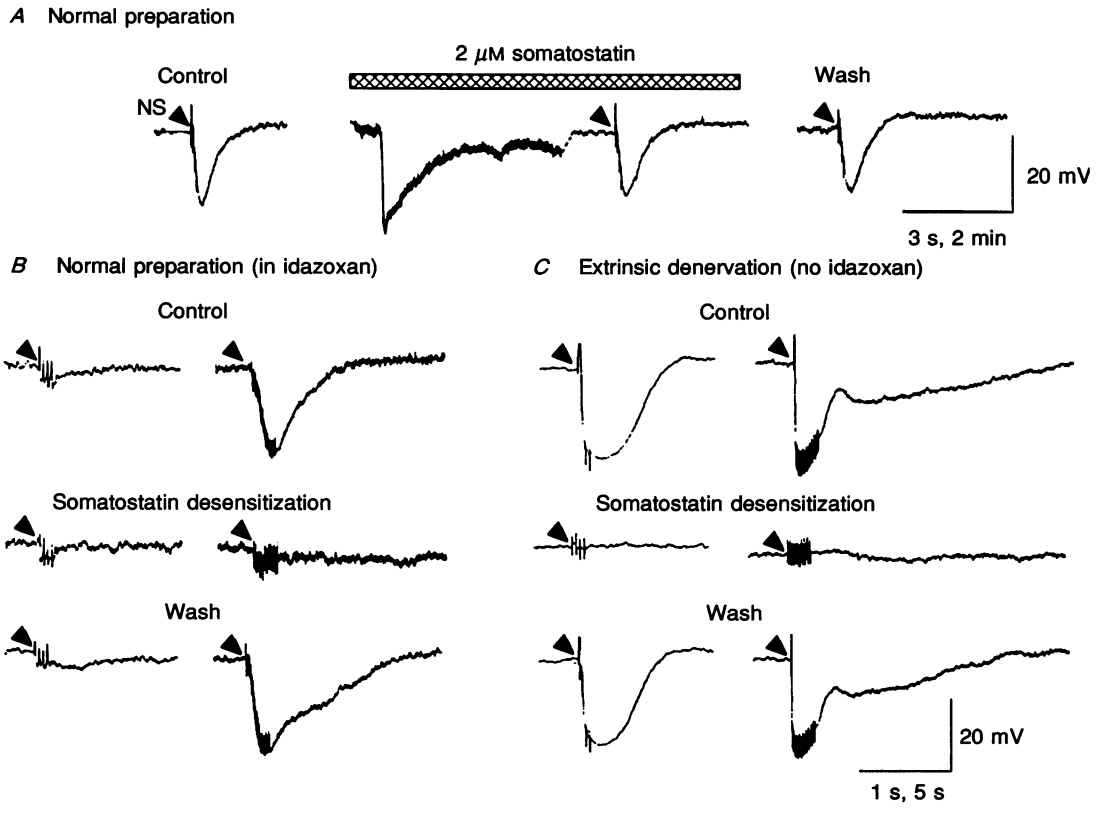

Fig. 9. Selective blockade of non-adrenergic IPSP by somatostatin desensitization. *A*, example of lack of effect of somatostatin desensitization on adrenergic IPSP recorded in response to 4 stimuli at 20 Hz in a normal preparation. Fast time calibration refers to recordings of IPSP; slow scale refers to recording during somatostatin superfusion. *B*, recordings obtained in one neurone from a normal preparation in the presence of idazoxan (2 μM) in response to stimulation at 20 Hz for 4 pulses (left-hand traces) or 40 pulses (right-hand traces). The non-adrenergic IPSP was evoked only by prolonged stimulation. Middle set of traces shows response evoked in the presence of somatostatin at a time when the hyperpolarization had faded to within 10 % of pre-drug resting potential (e.g. as in *A*); membrane potential held at original resting potential (−52 mV) in all cases. The non-adrenergic IPSP was abolished during somatostatin desensitization. *C*, recordings obtained from one neurone in an extrinsically denervated preparation, no adrenoceptor blockers present. Stimulation by 4 (left-hand traces) and 40 (right-hand traces) pulses evoked IPSPs, both of which were abolished during somatostatin desensitization (middle set of traces). NS, nerve stimulation (at symbol in each trace).

unaltered by the nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 100 μM; $n = 6$). The average amplitudes of the non-adrenergic IPSPs at −50 mV in normal and extrinsically denervated preparations in response to forty stimuli at 20 Hz were 17 ± 1 mV ($n = 9$) and 24 ± 1 mV ($n = 11$); these values were significantly different ($P = 0{\cdot}0002$). A direct comparison between time courses of

these IPSPs was not possible because the non-adrenergic IPSP in normal preparations was generally monophasic while that recorded from extrinsically denervated preparations was clearly biphasic, but the total duration of the IPSP (10–30 s) evoked by prolonged stimulation in denervated preparations was 3–10 times longer than the non-adrenergic IPSP in normal preparations (Fig. 9).

Effects of somatostatin desensitization

The adrenergic IPSP, like the noradrenaline pressure-ejection response (e.g. Fig. 4*B*), was unaltered when it was evoked in the presence of a concentration of somatostatin (2 μM) which produced marked tachyphylaxis of the somatostatin response (Fig. 9*A*). There were also no differences in the amplitudes of the nicotinic fast EPSP and the slow EPSP when they were evoked in control solution or in the presence of somatostatin (2 μM) at a time when the somatostatin response had desensitized ($n = 12$). However, the non-adrenergic IPSP evoked from normal preparations (Fig. 9*B*, $n = 12$) or from extrinsically denervated preparations (Fig. 9*C*, $n = 7$) was abolished during the tachyphylaxis produced by somatostatin.

DISCUSSION

Results from the present study extend previous observations on the adrenergic and non-adrenergic IPSP in submucosal neurones and strengthen the conclusion that the non-adrenergic IPSP is produced by the release of somatostatin from intrinsic myenteric neurones. Our results also suggest that somatostatin release from these neurones dramatically increases within a few days of removal of their extrinsic sympathetic innervation. Such conclusions have significant physiological implications with regard to pathological states of gastrointestinal secretory function.

The somatostatin response

Pharmacological and molecular cloning techniques have identified two distinct somatostatin receptors (Raynor & Reisine, 1989; Kluxen, Bruns & Lubbert, 1992; Yamada, Post, Wang, Tager, Bell & Seino, 1992; Li, Forte, North, Ross & Snyder, 1992); the cloned somatostatin-2 receptor ($SRIF_2$-R) appears to represent the pharmacologically characterized $SRIF_1$ receptor which is activated preferentially by very low concentrations of MK 678 (Raynor & Reisine, 1989; Raynor, Wang, Dichter & Reisine, 1991; Yamada *et al.* 1992). The somatostatin receptor underlying the potassium conductance increase in submucosal neurones falls into this subtype category based on our results with MK 678 ($EC_{50} = 0{\cdot}9$ nM) and other somatostatin analogues (EC_{50} values from 9 to 15 nM, Fig. 1).

Long-term (4–24 h) pretreatment with somatostatin results in a prolonged inhibition of somtatostatin receptor coupling to adenylate cyclase in pituitary cells (Reisine & Takahashi, 1984; Reisine, 1985). This desensitization of the somatostatin response is manifested by a 20- to 100-fold rightward shift in the somatostatin concentration–response curve rather than by abolition of the response; this form of desensitization also requires protein synthesis (Reisine & Takahashi, 1984). In contrast, desensitization of somatostatin coupling to potassium conductance activation in submucosal neurones occurred within 1–2 min of

application of high (2 μM) concentrations and was manifested as a complete block of the somatostatin response during the continued presence of somatostatin. Recovery of the somatostatin-induced potassium conductance increase was rapid and followed the time course of drug wash-out; the tachyphylaxis of the somatostatin response was related directly to the presence of high concentrations of somatostatin at the receptor. These observations, and the findings that activators of adenylate cyclase or protein kinase C, and inhibitors of membrane protein translocation did not alter desensitization, make it unlikely that A kinase, C kinase pathways and/or receptor internalization are responsible for the somatostatin receptor desensitization observed in the present study.

Two general types of receptor desensitization have been defined, heterologous desensitization where desensitization of a response to one agonist results in decreased sensitivity of that response to any number of other agonists, and homologous densitization which is agonist specific and does not result in loss of responsiveness to other agonists (Lefkowitz & Carron, 1987; Sibley *et al.* 1988). Obviously, heterologous desensitization is of little practical use as a tool to elucidate a transmitter function for a neurally contained substance. In the present study, desensitization of the somatostatin response did not alter the α_2-adrenoceptor or the opiate receptor coupling to potassium conductance activation. Previous studies have shown that all three receptors couple to the same potassium channels in an excised membrane patch (Shen *et al.* 1992), and that coupling in each case involves pertussis toxin-sensitive G-proteins (Mihara *et al.* 1987 *b*; Surprenant & North, 1988; Shen *et al.* 1992). Moreover, the potassium conductance increase and its coupling mechanism underlying the adrenergic and non-adrenergic IPSP are identical, and identical to that activated by the three inhibitory receptors (Surprenant & North, 1988; Mihara *et al.* 1989). Therefore, the homologous type of receptor desensitization by somatostatin observed in the present study provides an important tool for the discrimination of somatostatin as the neurotransmitter underlying the non-adrenergic IPSP.

Adrenergic and non-adrenergic IPSPs

Selective stimulation of the extrinsic nerve fibres (e.g. Fig. 6) directly demonstrated that noradrenaline released from sympathetic nerves mediates the adrenergic IPSP. Our results also indicate that only noradrenaline provides a functional synaptic input into submucosal neurones from the sympathetic nerves because all responses to selective sympathetic stimulation were abolished by α_2-adrenoceptor antagonists. Somatostatin is contained in these same sympathetic nerve fibres (Costa & Furness, 1984). Therefore, it might be expected that if somatostatin is indeed the transmitter underlying the non-adrenergic IPSP (see below), then high-frequency stimulation of these sympathetic fibres should evoke a non-adrenergic IPSP. However, we do not know whether somatostatin is released from these fibres in response to our method of stimulation, or whether somatostatin receptors are present at the same synaptic sites that possess α_2-adrenoceptors. Moreover, there are numerous examples where two (or more) substances are known to be released from nerve terminals onto cells possessing receptors for both

substances, and yet only one of the substances evokes a postsynaptic potential (see reviews by Burnstock, 1986, 1990).

α_2-Adrenoceptor antagonists decreased the amplitude and time course of the IPSP evoked in normally innervated submucosal neurones (see also North & Surprenant, 1985). As is to be expected for competitive receptor antagonists, higher concentrations of idazoxan and phentolamine were required to maximally inhibit the IPSP evoked in response to prolonged stimulation (20 Hz for 2 s) than that evoked in response to brief stimuli (20 Hz for 0·2 s). Maximally effective concentrations of idazoxan produced 95–100 % inhibition of the IPSP evoked by brief stimuli but only 50 % inhibition of the IPSP in response to prolonged stimuli; phentolamine produced maximum inhibitions of 88 and 48 % respectively (Fig. 7). These results confirm previous studies demonstrating a non-adrenergic IPSP in normally innervated preparations in response to high-frequency stimulation (Mihara *et al.* 1987 *b*; Bornstein *et al.* 1988) and provide a quantitative clarification of concentrations of antagonist required to abolish the adrenergic IPSP. Conclusions from a previous study suggested the non-adrenergic IPSP in response to brief stimuli was significantly faster than the adrenergic IPSP (Bornstein *et al.* 1988); this conclusion was based on comparisons of IPSPs in the absence and presence of 200 nM phentolamine, a concentration we found to inhibit the adrenergic IPSP by only 65 % (Fig. 7). In the present study, a comparison of the adrenergic IPSP in normal preparations and the non-adrenergic IPSP recorded from extrinsically denervated preparations in response to brief stimuli revealed no significant differences in amplitude or time course of these synaptic potentials.

The non-adrenergic IPSP recorded in the presence of maximal concentrations of idazoxan in normal preparations, as well as the non-adrenergic IPSP recorded from extrinsically denervated preparations, were selectively abolished by concentrations of somatostatin that produced desensitization of the somatostatin hyperpolarization. The time course of onset of the inhibition and recovery of the non-adrenergic IPSP paralleled the onset and offset of desensitization of the somatostatin response. There was no cross-desensitization of the adrenergic IPSP by somatostatin, nor any effect on the fast or slow EPSPs when they were evoked in the presence of desensitizing concentrations of somatostatin. Much previous evidence (outlined in the Introduction) exists for the presence, synthesis and release of somatostatin onto submucosal neurones, for a somatostatin mimicry of the IPSP and for the abolition of the non-adrenergic IPSP when all somatostatin-containing fibres are selectively removed. Selective antagonism is a further essential criterion for establishing a neurotransmitter role but previous studies have not demonstrated a selective antagonist for the non-adrenergic IPSP. We suggest that the selective blockade of the somatostatin response and the non-adrenergic IPSP by the homologous-type desensitization of the somatostatin receptor response characterized in the present study provides strong evidence that somatostatin is the neurotransmitter underlying this synaptic potential in submucosal neurones from both normal and extrinsically denervated preparations.

The appearance of the prominent non-adrenergic IPSP within 5–7 days of removal of the extrinsic sympathetic nerves observed in the present and previous (Bornstein *et al.* 1988) studies makes it unlikely that axonal sprouting and

reinnervation by somatostatin-containing fibres onto submucosal neurones can explain this observation. Immunohistochemical observations showed no increase in numbers of somatostatin-containing fibres projecting onto submucosal neurones 7 days after extrinsic denervation (M.-M. Jiang & A. Surprenant, personal observations). Previous studies have shown that axonal sprouting of substance P-containing myenteric neurones onto submucosal arterioles and neurones following extrinsic denervations occurs only after approximately 20–40 days (Galligan, Costa & Furness, 1988; Jiang & Surprenant, 1992). It seems more likely that removal of the sympathetic innervation induces an increased synthesis and/or release of somatostatin from neurones already in place at the synapse between somatostatin-containing nerve terminal and submucosal neurone.

Physiological significance

Submucosal neurones function to modulate mucosal water and electrolyte transport; their activation leads to increased net secretory activity while their inhibition leads to increased net absorption (Cooke, 1987; Keast, 1987). Sympathetic nerves, via the adrenergic IPSP, provide a tonic inhibition to submucosal neurones (see Surprenant, 1989). Several pathophysiological conditions, most notably diabetic neuropathies, result in destruction of sympathetic nerves to the gastrointestinal tract and consequent diarrhoeal states (Ogbonnaya & Arem, 1990); somatostatin analogues have proved useful in treatment of this type of diabetic diarrhoea (Dudl, Anderson, Forsythe, Ziegler & O'Dorisio, 1987; Gaginella, O'Dorisio, Fassler & Mekhjian, 1990). In man, and in animal models of autonomic neuropathies, this type of diarrhoea has often been observed to reverse spontaneously but it is not clear how this might occur (Ogbonnaya & Arem, 1990). Results from the present study suggest one potential mechanism that might be involved in the plastic reversal of sympathectomy-induced increase in mucosal secretory activity. That is, it might be that removal of sympathetic innervation to enteric neurones induces an increased synthesis and/or release of somatostatin from intrinsic nerves. Because the actions of somatostatin are identical to those of noradrenaline on enteric neurones, somatostatin may effectively replace sympathetic noradrenaline as the major inhibitory neurotransmitter to submucosal neurones under pathological conditions associated with autonomic neuropathies.

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