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A NON-ADRENERGIC, NON-CHOLINERGIC SLOW INHIBITORY POST-SYNAPTIC POTENTIAL IN NEURONES OF THE GUINEA-PIG SUBMUCOUS PLEXUS

BY S. MIHARA*, S. NISHI*, R. A. NORTH† AND ANNMARIE SURPRENANT†

*From the † Neuropharmacology Laboratory, 56–245 Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A. and the * Department of Physiology, Kurume University Medical School, 67 Asahi-machi, Kurume 830, Japan*

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SUMMARY

1. Intracellular recordings were made from neurones in the submucous plexus of guinea-pig ileum and caecum. The responses to electrical stimulation of fibre strands entering the nodes of the plexus were studied.

2. Stimuli comprising trains of pulses (20 Hz, 1–5 s) produced nicotinic excitatory post-synaptic potentials (fast e.p.s.p.s), an adrenergic inhibitory post-synaptic potential (i.p.s.p.), a slow excitatory post-synaptic potential (slow e.p.s.p.) and a fourth, hitherto unnoticed, slow hyperpolarization which followed the slow e.p.s.p. All these responses were abolished by tetrodotoxin or solutions containing a low calcium concentration.

3. The slow hyperpolarization (slow i.p.s.p.) was examined in the presence of blockers of the nicotinic and adrenergic responses, and in conditions in which the slow e.p.s.p. was prevented by desensitizing concentrations of substance P or vasoactive intestinal polypeptide. The slow i.p.s.p. was unaffected by prazosin (0·1–1 μM), propranolol (0·1–1 μM), atropine (1 μM) or naloxone (1 μM).

4. The amplitude and duration of the slow i.p.s.p. increased with increasing numbers of stimulus pulses; it had an amplitude of 17 mV and a duration of 70 s when evoked by a stimulus of 20 Hz for 3 s.

5. The slow i.p.s.p. was associated with a decrease in the input resistance of the cell. It reversed polarity at −90 mV in 4·7 mM-potassium and the extrapolated reversal potential in 0·47 mM-potassium was −145 mV; these findings indicate that the slow i.p.s.p. results from an increase in membrane potassium conductance.

6. The slow i.p.s.p. could still be recorded from submucous plexus neurones in segments of ileum which had been extrinsically denervated 6–11 days previously.

7. The results demonstrate the existence of a distinct synaptic potential, the slow i.p.s.p., which is due to release of a non-cholinergic, non-adrenergic transmitter from neurones whose cell bodies lie within the enteric plexuses.

† Present address: Institute for Advanced Biomedical Research, Oregon Health Sciences University, 3161 S.W. Sam Jackson Park Road, Portland, OR 97201, U.S.A.

INTRODUCTION

A variety of synaptic potentials have been described among the neurones of the autonomic nervous system, and in some cases the transmitter responsible for them has been identified with reasonable confidence. Few dispute that the fast excitatory post-synaptic potential (e.p.s.p.) is mediated by acetylcholine acting on nicotinic receptors, whereas an action of acetylcholine on a type of muscarinic receptor is responsible for a slow e.p.s.p. brought about by closure of membrane potassium channels (see North, 1986). Hyperpolarizing synaptic potentials have been described which result from an increase in potassium conductance; either acetylcholine (Hartzell, Kuffler, Stickgold & Yoshikami, 1977; Dodd & Horn, 1983) or noradrenaline (North & Surprenant, 1985) are responsible for these inhibitory post-synaptic potentials (i.p.s.p.s). Muscarinic e.p.s.p.s or i.p.s.p.s and adrenergic i.p.s.p.s typically last for 1–10 s, but in many neurones they are followed by a longer lasting depolarizing synaptic potential which was called the late slow e.p.s.p. by Nishi & Koketsu (1968) when they first observed it in bull-frog sympathetic ganglion cells. This non-cholinergic, non-adrenergic e.p.s.p. appears to be mediated by a variety of different transmitters in different preparations, such as substance P (some guinea-pig enteric neurones, inferior mesenteric ganglion), 5-hydroxytryptamine (guinea-pig coeliac ganglion) or a compound related to luteinizing hormone releasing hormone (bull-frog sympathetic ganglion; for review see North, 1986).

A number of peptides contained within autonomic nerves hyperpolarize neurones when they are applied exogenously, but the question remains whether they mediate synaptic potentials. Somatostatin (Mihara, North & Surprenant, 1987) and enkephalin (Mihara & North, 1986) both hyperpolarize neurones of the guinea-pig submucous plexus, and both are contained within fibres terminating around submucous plexus neurones. The somatostatin is contained within fibres having their origin in the prevertebral ganglia (where it coexists with noradrenaline; Costa & Furness, 1984) as well as fibres with cell bodies in the myenteric plexus or the submucous plexus (Costa, Furness, Llewellyn-Smith, Davies & Oliver, 1980). Indeed, about 17% of cell bodies in the submucous plexus contain somatostatin-like immunoreactivity, and these cells send their fibres not only to the mucosa but also to other submucous plexus neurones, where they make basket-like arrangements around them. Enkephalin is contained in myenteric but not submucous plexus neurones; however, one of the several defined projections of the enkephalin-containing fibres is to the cells of the submucous ganglia (Furness & Costa, 1986). The presence of these peptides within fibres entering submucous ganglia, the varicose clutches of the fibres encircling submucous plexus neurones, and the rather well-characterized post-synaptic actions of these two peptides when applied exogenously, set the stage for the present experiments.

METHODS

The methods of intracellular recording and application of drugs have been described (Surprenant, 1984; Mihara *et al.* 1987). Nerve fibre bundles running in the submucous plexus were stimulated electrically by passing brief currents through an electrode which contained the superfusing solution, the tip of which (diameter 10–30 μm) was positioned less than 20 μm from the bundle. The stimuli

applied comprised either single pulses (typically 100–700 μs duration) or trains of pulses (ranging from 5 to 200 pulses at 5–20 Hz).

The results described are based on intracellular recordings from neurones in the caecum. A smaller number ($n = 11$) of experiments on the guinea-pig ileum gave qualitatively similar results. The experiments on the ileum were carried out so that the results could be compared with those obtained on tissues extrinsically denervated (it is very difficult to denervate extrinsically the caecum). Denervation was performed in eight guinea-pigs under etorphine and Nembutal anaesthesia, by crushing the mesenteric nerves running to the intestine with watchmaker's forceps 6–11 days prior to the *in vitro* experiment (Furness & Costa, 1978). The success of the denervation was assessed by examining the tissue for catecholamine fluorescence using the formaldehyde–glutaraldehyde method of Furness, Costa & Wilson (1978).

RESULTS

The effects of stimulating the presynaptic nerves with a single pulse or with a train of up to five pulses were similar to those previously described. The fast e.p.s.p. (or series of up to five fast e.p.s.p.s) was followed by an i.p.s.p., which was followed by a slow e.p.s.p. The i.p.s.p. under these conditions was typically 1 s in duration; the slow e.p.s.p. lasted for 10–30 s (see Surprenant, 1984; Mihara, Katayama & Nishi, 1985). When the number of pulses applied was increased by increasing the duration of the stimulus, the slow e.p.s.p. became longer (see Surprenant, 1984); when more than ten pulses were applied the later part of the slow e.p.s.p. was replaced by a membrane hyperpolarization (Fig. 1).

The i.p.s.p. that immediately followed the fast e.p.s.p.(s) could be completely blocked by adding idazoxan to the superfusing solution (Fig. 2). A concentration of 1 μM was usually used, which is about 100 times the dissociation equilibrium constant for the α_2-receptor (North & Surprenant, 1985). Yohimbine had a similar effect. In order to study the slower hyperpolarizing response in isolation, it was desirable to block the slow e.p.s.p. No antagonists are available, hence advantage was taken of the finding by Mihara *et al.* (1985) that superfusion with substance P or vasoactive intestinal polypeptide (VIP) blocked the slow e.p.s.p. while leaving unaffected the i.p.s.p. and fast e.p.s.p. Under these circumstances, repetitive stimulation of presynaptic nerves elicited a slow hyperpolarizing response which was graded with the number of pulses applied (Fig. 2). This slow hyperpolarizing response was reversibly abolished by tetrodotoxin (500 nM) or by solutions containing a low (0·25 mM) calcium concentration. It was unaffected by prazosin (up to 1 μM), propranolol (up to 1 μM), (+)-tubocurarine (50 μM), hexamethonium (500 μM), atropine (1 μM) or naloxone (1 μM).

The slow synaptic hyperpolarization could be evoked in almost all neurones (forty-eight of fifty-two) which had fast e.p.s.p.s and adrenergic i.p.s.p.s, provided that a sufficiently long presynaptic stimulus was applied (more than ten pulses were usually necessary). The mean amplitude of the synaptic hyperpolarization was $17{\cdot}1 \pm 0{\cdot}7$ mV ($n = 48$), and the mean duration was $69{\cdot}8 \pm 3{\cdot}5$ s ($n = 48$), when it was evoked by a simulus of 20 Hz for 3 s, in the presence of idazoxan (300 nM or 1 μM) and substance P (400 nM). No similar prolonged hyperpolarization followed a train of sixty action potentials (at 20 Hz) which was evoked by passing a series of depolarizing current pulses through the micro-electrode (Fig. 3). The synaptically evoked hyperpolarization was often clearly biphasic (e.g. Fig. 3); it is possible that the initial

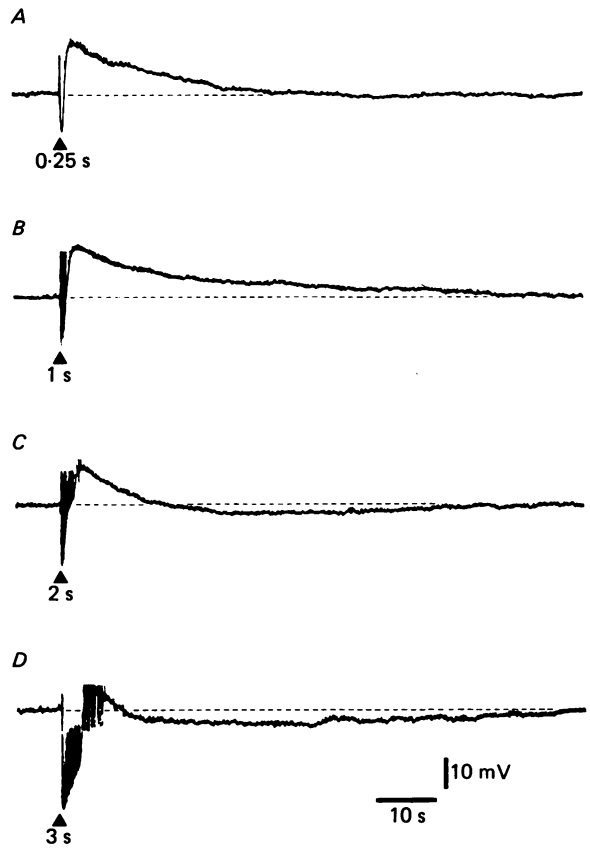

Fig. 1. Synaptic potentials evoked by repetitive nerve stimulation at 20 Hz for various durations, as indicated. *A*, five pulses evoked fast e.p.s.p.s followed by the biphasic slow response consisting of the i.p.s.p. and slow e.p.s.p. *B*, the slow e.p.s.p. was prolonged by stimulation for 1 s. *C*, stimulation for a 2 s period now produced a small hyperpolarization following the slow e.p.s.p. *D*, this slow hyperpolarization increased in amplitude and duration with a 3 s stimulation period. Note that the amplitude and time course of the slow e.p.s.p. decreased at these stimulation parameters (*C* and *D*). Action potentials associated with the fast and slow e.p.s.p.s are attenuated by the low-frequency response of the pen recorder.

rapidly decaying component resulted from a persisting adrenergic response which was not blocked by the α_2-antagonist present, since it was not observed in preparations from denervated segments of ileum (see below).

In a smaller number of similar experiments carried out on ileal neurones, the slow synaptic hyperpolarization was recorded from eight of fourteen neurones. A stimulus of 20 Hz for 3 s evoked a slow i.p.s.p. of 10 ± 1 mV in amplitude and 40 ± 6 s in duration; these recordings were obtained in idazoxan (2 μM) and substance P (2 μM).

The slow synaptic hyperpolarization was associated with a reduced cell input resistance (Fig. 3). The effect of changing the potassium ion concentration was examined. An increase in the potassium concentration to 10 or 20 mM depressed or abolished the synaptic response; this may be due to depolarization blockade of the

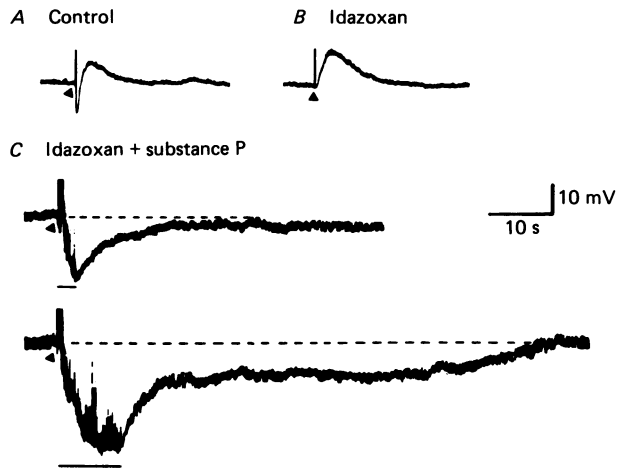

Fig. 2. Slow i.p.s.p. isolated by blockade of adrenergic i.p.s.p. and slow e.p.s.p. *A*, typical triphasic response to presynaptic nerve stimulation (20 Hz, 250 ms) comprising initial fast e.p.s.p.s (not resolved at this recording speed) followed by a hyperpolarizing i.p.s.p. and a slow e.p.s.p. *B*, idazoxan (300 nM) selectively blocked the i.p.s.p. *C*, in a solution containing both idazoxan (300 nM) and substance P (400 nM), an increase in the number of presynaptic stimulus pulses evokes a slow i.p.s.p. Top trace in *C* shows the response to a train of 20 Hz for 2 s; bottom trace is response to 20 Hz for 10 s. All records are from the same neurone.

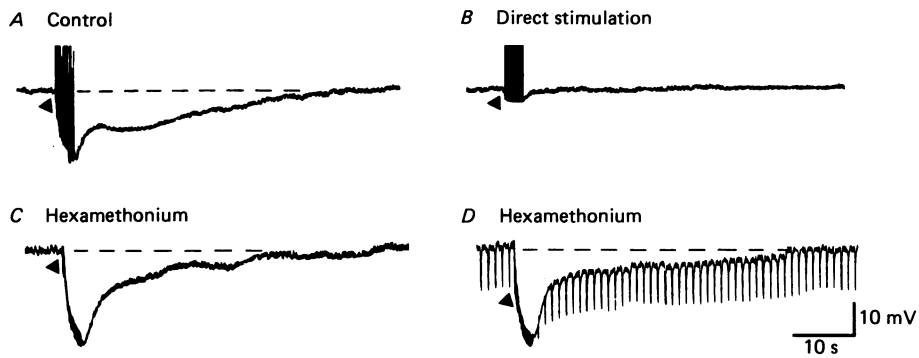

Fig. 3. *A*, slow i.p.s.p. recorded in the presence of idazoxan (300 nM) and substance P (400 nM) evoked by presynaptic stimulation (20 Hz for 3 s). Fast e.p.s.p.s giving rise to action potentials occurred but are not resolved at this recording speed. Note the clearly biphasic slow i.p.s.p. *B*, a train of action potentials was evoked by depolarizing pulses passed through the recording electrode (20 Hz for 3 s). The action potentials were not followed by any long-lasting hyperpolarization. *C*, hexamethonium (200 μM) blocked the fast e.p.s.p.s but did not affect the slow i.p.s.p. *D*, the slow i.p.s.p. is associated with a fall in input resistance. Downward deflections are electrotonic potentials evoked by passing current pulses (100 pA, 100 ms duration, 1 Hz) through the recording electrode. Hexamethonium (200 μM) present.

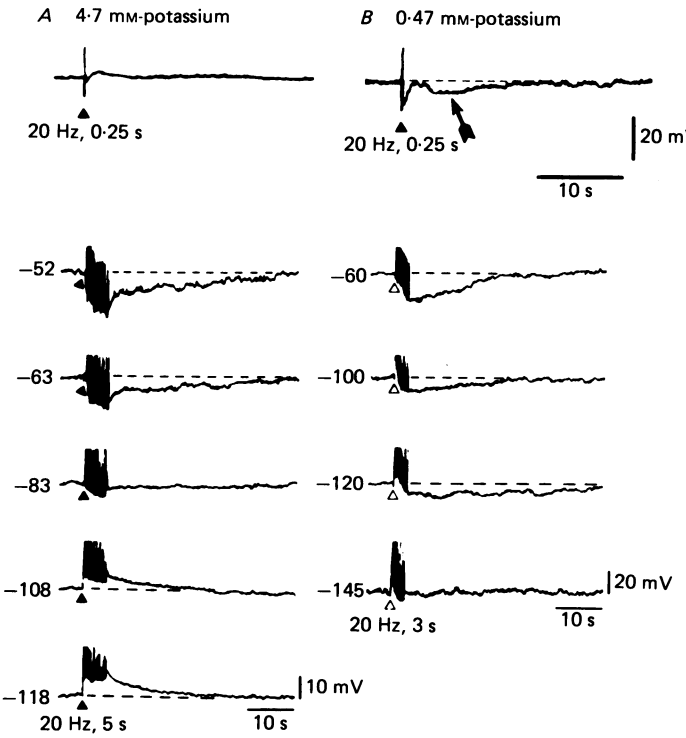

Fig. 4. Slow i.p.s.p. results from an increase in potassium conductance. Yohimbine (1 μM) and substance P (500 nM) were present throughout. *A*, in 4·7 mM-potassium a brief train of presynaptic stimuli (20 Hz for 0·25 s) had little effect except to produce the fast e.p.s.p.s and a small slow e.p.s.p. When the stimulus was increased to 20 Hz for 5 s, a slow i.p.s.p. became apparent. Hyperpolarization of the membrane progressively reduced the amplitude and eventually reversed the polarity of the slow i.p.s.p. at about −90 mV. *B*, the potassium concentration was reduced to 0·47 mM. In this condition even a short presynaptic stimulus (20 Hz for 0·25 s) evoked a small slow i.p.s.p. Increasing the stimulus strength to 20 Hz for 3 s resulted in a large slow i.p.s.p. which reversed polarity at −145 mV. Numbers beside records indicate membrane potential in millivolts. Note the different scales in the upper and lower sets of records.

presynaptic fibres (Johnson, Katayama & North, 1980; Surprenant, 1984). However, in solutions containing only 0·47 mM-potassium (one-tenth normal), the synaptic hyperpolarization was markedly augmented in amplitude. Indeed, in low potassium concentrations the synaptic hyperpolarization was often prominent following only a few shocks to the presynaptic nerves (Fig. 4). Conditioning hyperpolarization of the membrane reduced the amplitude of the synaptic potential, and it reversed polarity at $-90{\cdot}8 \pm 1{\cdot}4$ mV ($n = 5$) (Fig. 4); in a solution containing a low concentration of potassium ions this reversal potential changed to $-146{\cdot}1 \pm 1{\cdot}7$ mV ($n = 3$). These values are not different from those found for the reversal of the hyperpolarizations (or currents) evoked by somatostatin (Mihara *et al.* 1987) or enkephalin (Mihara & North, 1986). It seems unlikely that chloride conductance changes contributed significantly to the response, both because of the close to Nernstian shift in reversal potential when the potassium concentration was altered, and because the responses

remained unchanged in amplitude throughout long periods of recording (up to 5 h) with potassium chloride-filled electrodes.

The submucous plexus from all eight guinea-pigs in which a segment of ileum was extrinsically denervated showed a marked loss of catecholamine fluorescence; however, in only three experiments was fluorescence completely absent in the tissue containing the nodes of plexus from which intracellular recordings were made. Neurones in these three preparations showed typical fast e.p.s.p.s and slow e.p.s.p.s, but no i.p.s.p. following one to three pulses to the internodal fibre strands ($n = 18$). All of these neurones were hyperpolarized by application of noradrenaline and/or somatostatin (by superfusion or by application from a blunt pipette). A train of pulses (20 Hz for 1–3 s) evoked a slow synaptic hyperpolarization in eight of eighteen neurones, and this had the characteristics of the slow i.p.s.p. recorded from control animals (Fig. 5). This slow i.p.s.p. was unaffected by very high concentrations of idazoxan (20 μM) or naloxone (1 μM).

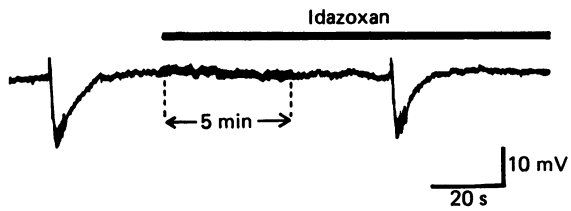

Fig. 5. Slow i.p.s.p. recorded from ileal submucous plexus neurone after extrinsic denervation. The slow i.p.s.p. was evoked by stimulation at 20 Hz for 4 s before and approximately 5 min after the addition of 20 μM-idazoxan. Hexamethonium (100 μM) was present in order to block the fast e.p.s.p.; no slow e.p.s.p. was present in this neurone. No catecholamine fluorescence was observed from the preparation in which this recording was obtained. Naloxone (1 μM) was present throughout.

DISCUSSION

The main point of this paper is the description of a previously unrecognized synaptic potential in neurones of the guinea-pig submucous plexus. The ionic mechanism of this inhibitory synaptic potential appears to be that of a potassium conductance increase, as manifest by its reversal potential at the potassium equilibrium potential and its approximately Nernstian dependence on external potassium at the two concentrations we were able to examine reliably. The slow i.p.s.p. described in this paper can be differentiated from the adrenergic i.p.s.p. by its time course which is 10–100 times slower than the adrenergic i.p.s.p. and by its insensitivity to α_2-adrenoceptor blockers. It is difficult to exclude completely a contribution of noradrenaline to the initial part of the slow i.p.s.p. because the repetitive nerve stimulation might release more transmitter than can be blocked even by the high concentrations of antagonist used. Therefore, more reliance must be placed on the continued presence of the slow i.p.s.p. after extrinsic denervation. The first description of this synaptic potential may well be that of Hirst & McKirdy (1975), who found that synaptic hyperpolarizations could be evoked in preparations of guinea-pig ileum which had been extrinsically denervated. Acetylcholine can also be

ruled out as a transmitter underlying the slow i.p.s.p. because nicotinic and muscarinic receptor antagonists did not alter this synaptic potential.

Of the other substances known to be present within nerves ramifying in the submucous ganglia, two have actions which mimic the slow i.p.s.p. These are enkephalin and somatostatin (Mihara & North, 1986; Mihara *et al.* 1987). A role for enkephalin seems unlikely because naloxone did not inhibit the slow i.p.s.p.; however, the naloxone concentration was only about 50 times the dissociation constant for the δ-receptor (Mihara & North, 1986). A role for somatostatin remains a distinct possibility. If somatostatin is responsible for the slow i.p.s.p. it would appear to be released primarily from intrinsic nerve fibres rather than from sympathetic fibres. In any event, the finding that the slow i.p.s.p. could still be recorded after extrinsic denervation implies that a substance contained within intrinsic nerve fibres is the transmitter.

Other substances which are known to be within the intrinsic nerves include VIP, substance P, calcitonin gene-related peptide (CGRP), 5-hydroxytryptamine (5-HT), cholecystokinin (CCK), neuropeptide Y and galanin (Furness, Costa & Keast, 1984; Furness, Costa, Gibbins, Llewellyn-Smith & Oliver, 1985; Furness & Costa, 1986). Substance P, 5-HT, VIP and CCK only depolarize submucous plexus neurones (Hirst & Silinsky, 1975; Neild, 1978; Surprenant, 1984; Mihara *et al.* 1985). CGRP also depolarizes these cells whereas neuropeptide Y and galanin do not alter the resting potential (J. Crist & A. Surprenant, unpublished observations). Thus, of the substances presently known to be contained within the submucous nerves, somatostatin remains the front-running candidate for the mediator of the slow i.p.s.p.

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