Journal of Physiology (1993), **462**, *pp*. 483–501
With 7 figures
Printed in Great Britain

COMMON IONIC MECHANISMS OF EXCITATION BY SUBSTANCE P AND OTHER TRANSMITTERS IN GUINEA-PIG SUBMUCOSAL NEURONES

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(*Received 27 March 1992*)

SUMMARY

1. Intracellular recordings were made from submucosal neurones and single-electrode voltage-clamp methods were used to record membrane currents. The actions of substance P (SP), 5-hydroxytryptamine (5-HT), muscarine, vasoactive intestinal polypeptide (VIP), forskolin and nerve stimulation were studied.
2. Substance P, 5-HT (in the presence of 5-HT_3 receptor antagonists), muscarine, VIP, forskolin and slow excitatory synaptic transmission all produced identical responses: an inward current associated with a membrane conductance decrease at the resting potential. The actions of any one occluded the actions of any other and all responses were pertussis-toxin insensitive.
3. These agonists produced a voltage-independent decrease in a 'leak' potassium conductance between −40 and −120 mV in 14% of neurones.
4. These agonists decreased a voltage-dependent, calcium-activated potassium conductance between −40 and −80 mV in all other (86%) neurones. The agonists still evoked an inward current without apparent conductance change at potentials between −90 and −130 mV.
5. In a low calcium solution containing cobalt or cadmium, the agonists produced an inward current associated with a conductance increase from −40 to −120 mV. Ion replacement studies indicated this current was due to an increase in a cation-selective (mainly sodium) conductance.
6. The agonists also reduced the inwardly rectifying potassium current that is activated by somatostatin and α_2-adrenoceptor agonists in these neurones. The agonists did not alter the inwardly rectifying potassium current that is present in these neurones in the absence of somatostatin or α_2-agonists.
7. Thus, SP, 5-HT, muscarine, VIP and the release of slow excitatory transmitters all appear to act through a common intracellular transduction pathway, an increase in adenylate cyclase. This results in an activation of a sodium-selective cation current and an inhibition of three distinct potassium conductances: the background potassium conductance, the calcium-activated potassium conductance and the inwardly rectifying potassium conductance activated by somatostatin and α_2-adrenoceptor agonists.

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INTRODUCTION

A single enteric neurone possesses a multitude of receptors whose activation results in a depolarization with an associated decrease in membrane conductance; these receptors include substance P (SP), muscarinic acetylcholine, vasoactive intestinal polypeptide (VIP), 5-hydroxytryptamine (5-HT), cholecystokinin (CCK), and adenosine (Furness & Costa, 1987; Surprenant, 1989; Tokimasa & Akasu, 1992). These substances are present in cell bodies and/or nerve fibres in the enteric plexuses and there is good evidence that acetylcholine (ACh), SP, VIP and 5-HT can be synaptically released (North, 1982; Furness & Costa, 1987). Brief, local application of these substances onto enteric neurones evokes a slow depolarization whose time course and underlying conductance change is similar to that of the slow excitatory postsynaptic potential (slow EPSP) (Katayama & North, 1978; Surprenant, 1984, 1989; North, Slack & Surprenant, 1985; Mihara, Katayama & Nishi, 1986; Surprenant & Crist, 1988; Mihara & Nishi, 1989).

It has been clear for a number of years that a decreased potassium conductance is primarily responsible for the slow EPSP and the depolarization produced by the above-noted substances (Katayama & North, 1978; Grafe, Mayer & Wood, 1980; Surprenant, 1984; Mihara *et al.* 1986). A voltage-independent background potassium conductance and a voltage-dependent calcium-activated potassium conductance have been shown to be targeted by the so-called slow excitatory transmitters (Katayama & North, 1978; Grafe *et al.* 1980; Surprenant, 1984; Mihara *et al.* 1986; Surprenant, North & Katayama, 1987; Akasu & Tokimasa, 1989; Morita & Katayama, 1992). There is good indirect evidence to suggest that activation of the 'cyclic AMP cascade' is the primary intracellular transduction process involved in closure of potassium channels by most of these substances (Nemeth, Palmer, Wood & Zafirov, 1986; Palmer, Wood & Zafirov, 1986, 1987; Akasu & Tokimasa, 1989). It is not known whether activation of each receptor present on a single enteric neurone will result in an identical cascade of intracellular transduction pathways and alterations in ionic conductances. Nor is it known whether other membrane conductances may be altered by any of the slow excitatory transmitters. One of the main aims of the present study was to investigate these questions by applying an array of 'slow excitatory' substances onto a single submucosal neurone and determining the ion conductances so affected.

Somatostatin, α_2-adrenoceptor and δ-opioid receptor agonists all hyperpolarize guinea-pig submucosal neurones by activating the same set of inwardly rectifying potassium channels (North & Surprenant, 1985; Mihara *et al.* 1986; Mihara, North & Surprenant, 1987; Surprenant & North, 1988; Shen, North & Surprenant, 1992). We were particularly interested in determining whether slow excitatory transmitters can inhibit the potassium conductance activated by inhibitory agonists in submucosal neurones. Therefore, we also examined the actions of SP, VIP, 5-HT, muscarine, forskolin and nerve stimulation on membrane currents recorded from guinea-pig submucosal neurones in the absence and presence of somatostatin or the α_2-adrenoceptor agonist, UK 14304.

METHODS

Guinea-pigs (200–300 g) were stunned and exsanguinated; this protocol has been approved by the Animal Care and Use Committee of the Oregon Health Sciences University. Detailed methods of dissection of submucosal plexus, intracellular recordings, nerve fibre stimulation and drug applications have been described in previous papers (Surprenant 1984; Mihara *et al.* 1987). A segment of ileum was removed about 10–20 cm proximal to the ileocecal junction and placed in a physiological saline of composition (mM): NaCl, 126; NaH_2PO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 2·5; KCl, 4·5; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2 and 5% CO_2. ICS 205–930 (1 μM) was present in all experiments in which 5-HT was applied to block 5-HT_3 receptors; ICS 205–930 was not present when other agonists were applied. A small piece of ileum (8–12 mm long) was cut open and pinned out on Sylgard, mucosal surface up. The mucosa was stripped with forceps, and submucous plexus with attached connective tissue was pulled away from the underlying circular smooth muscle. The preparation was pinned to the base of a small recording chamber (0·5 ml volume) with mucosa surface down. Only those preparations to which no visible circular smooth muscle strands adhered were used in these experiments. The recording chamber was placed on the stage of an inverted microscope and viewed at 200 or 400 × magnification. The superfusing solution flowed at 4 ml/min with temperature in the recording chamber maintained at 35–36 °C. Tetrodotoxin (TTX, 600 nM) was always added to the superfusion solution except when synaptic potentials were studied.

In the majority of experiments, drugs were applied by superfusion; complete exchange of solution in the bath required about 30–60 s. In some experiments, substances (SP and VIP, 10 μM) were ejected by pressure from a pipette with tip diameter of 2–5 μm. The tip of this pipette was placed within 10–30 μm of the impaled neurone. Duration of pressure (10 lb/in^2 (68 kPa)) pulses was 10 ms.

A focal stimulating electrode (tip diameter 20–45 μm) was placed onto one ganglion or onto several internodal strands. Intracellular recordings were made from neurones in the first or second ganglion in a line away from the site of stimulation.

Membrane potentials and currents were recorded with a microelectrode containing 2 M KCl (resistance 50–80 MΩ). Electrode resistance was compensated using an amplifier with an active bridge circuit for current-clamp recordings, or a single electrode voltage clamp with a sample-and-hold circuit (Axoclamp 2, Axon Instruments). For voltage-clamp recordings, a 3 kHz switching frequency, with 50% duty cycle, was typically used, and head stage voltage was monitored on a separate oscilloscope. Membrane potential and current were displayed directly using a pen recorder (Gould 2400S). Steady-state current–voltage (I–V) relations were usually constructed by measuring membrane current during slow (3 mV/s) ramp commands.

Steady-state current–voltage (I–V) curves were computed by varying any of five conductances represented by: (1) a leak potassium conductance ($g_{K,L}$) which was the basic Goldman–Hodgkin–Katz equation (see Hille, 1984), (2) an inward rectifier potassium conductance ($g_{K,IR}$) described by a Boltzman function centred around -100 mV with a slope (steepness) of 6 mV, (3) a calcium-activated potassium conductance ($g_{K,Ca}$) described by a Boltzman function of the opposite sign, centred at -50 mV with steepness of 20 mV, (4) an agonist-activated potassium conductance ($g_{K,agonist}$) having a Boltzman function centred at -50 mV with a slope of 20 mV and (5) an agonist-activated cation conductance (g_{cation}) with a reversal potential of 0 mV.

The following drugs were used: noradrenaline, tetrodotoxin, substance P, vasoactive intestinal peptide, somatostatin, muscarine, forskolin, 5-hydroxytryptamine, hexamethonium, anthracene-carboxylic acid (9-AC), dibutyryl cyclic AMP, 3-isobutyl-1-methylxanthine (IBMX) (Sigma); idazoxan (gift of Reckitt and Colman); pertussis toxin (islet-activating protein) was purchased from List Biological Laboratories. (3α-Tropanyl)*H*-indole-3-carboxylic acid ester (ICS 205–930) was a gift from Sandoz. Details of pertussis toxin pretreatment have been described previously (Shen & Surprenant, 1990).

Results were expressed as means ± S.E.M. and comparison between groups were by Student's *t* test; *n* refers to number of cells.

RESULTS

Intracellular recordings were obtained from 152 neurones in which impalements were maintained for 1–7 h. Electrophysiological and synaptic properties (resting potential, input resistance, membrane time constant, current–voltage relations and

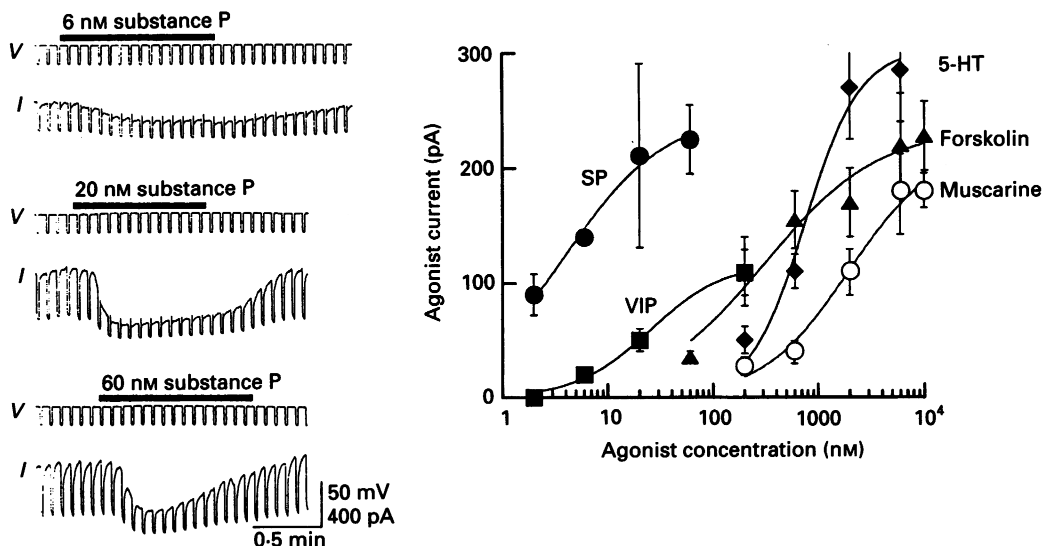

Fig. 1. Inward currents evoked by SP (*A*) and other slow excitatory agonists (*B*). *A*, inward current recorded at the resting potential (−53 mV) in response to superfusion with increasing concentrations of SP (duration of application indicated by filled bars). Downward deflections are currents to voltage command of 20 mV for 1 s at 0·25 Hz in this and subsequent figures. *B*, concentration–response curves for SP (●), VIP (■), 5-HT (◆), forskolin (▲) and muscarine (○) from all experiments. Peak currents were measured at the resting potential (−45 to −55 mV), $n = 3–12$ for each point.

presence of inhibitory and slow excitatory postsynaptic potentials (IPSPs and slow EPSPs) became stable only after 20–30 min and we were not able to adequately voltage clamp a neurone until approximately 45–60 min after impalement. Results reported herein were obtained from neurones (approximately 85% of all impalements) in which stimulation of the nerve supply evoked an adrenergic IPSP (see North & Surprenant, 1985) and slow EPSP in addition to the fast nicotinic EPSP. Thus, all cells examined in the present study can be considered to be 'S' cells (see Furness & Costa, 1987); further, they can all be considered to be VIP-containing neurones (Bornstein & Furness, 1988).

Excitation by substance P and other slow excitatory agonists

Substance P and forskolin evoked an inward current in all neurones ($n = 60$); 5-HT (in the presence of 1 μM ICS 205–930 to block 5-HT_3 receptors, see Surprenant & Crist, 1988) produced an inward current in 70% of cells ($n = 26$), muscarine was effective in 55% ($n = 30$) and VIP in only 25% ($n = 25$) of cells.

Time course. In agreement with previous studies (Mihara *et al.* 1986; Surprenant *et al.* 1987; Surprenant & Crist, 1988), brief (10 ms duration) pressure application of SP, VIP, 5-HT, or muscarine mimicked the slow EPSC in amplitude, time course and conductance change measured at the resting potential. For example, in the presence

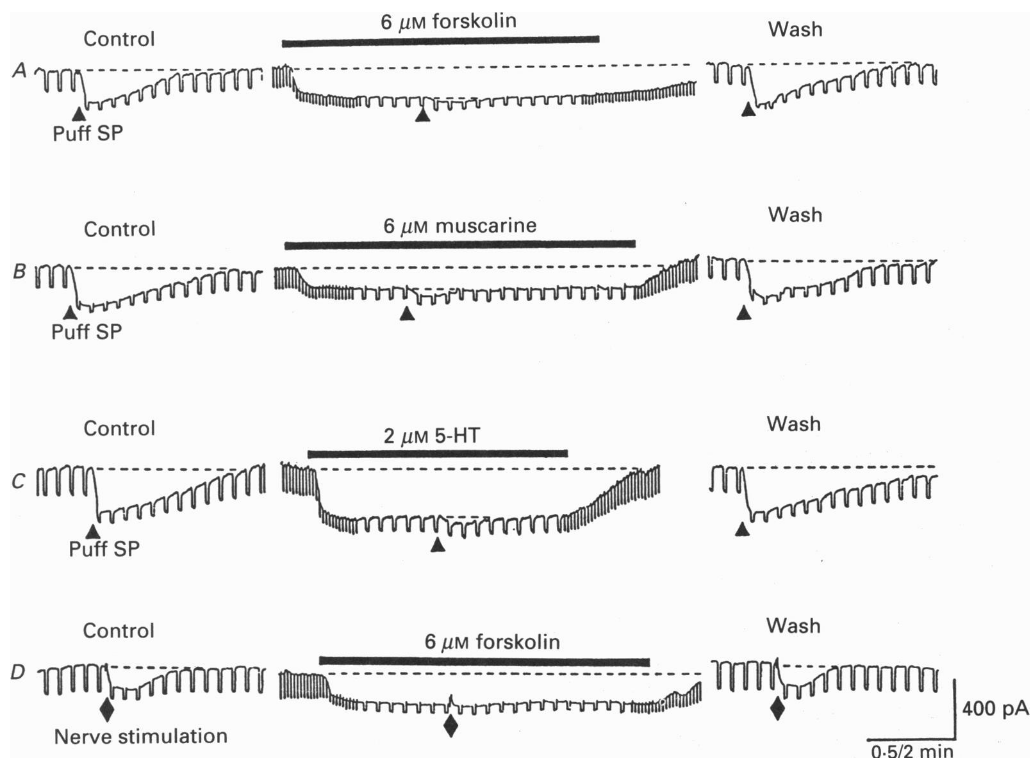

Fig. 2. Occlusion of SP current (▲) and the slow EPSC (◆) during superfusion with forskolin (*A* and *D*), muscarine (*B*), and 5-HT (*C*). SP was applied by pressure ejection, nerve stimulation (5 pulses at 20 Hz) applied in the presence of hexamethonium and idazoxan to block fast nicotinic EPSCs and adrenergic IPSC. All recordings obtained at the resting potential (−47 mV). Faster time calibration (0·5 min) refers to all recordings during puff application of agonist; slower time calibration (2 min) refers to beginning and end of traces during superfusion with agonist.

of 100 μM hexamethonium and 300 nM idazoxon to block the nicotinic EPSP and the adrenergic IPSP, nerve stimulation at 10 Hz for 0·5 s evoked a slow EPSP of 240 ± 54 pA with half-duration of 15 ± 3 s ($n = 9$). Pressure application of SP evoked an inward current of 230 ± 46 pA with half-duration of 24 ± 4 s ($n = 10$); pressure pulses of 5-HT or muscarine evoked inward currents of similar amplitude and half-durations (5-HT 310 ± 45 pA, 15 ± 4 s, $n = 6$; muscarine 190 ± 25 pA, 39 ± 6 s, $n = 4$).

Inward currents recorded at the resting potential in response to concentrations of SP between 2 and 20 nM reached peak amplitude within 20 s and were maintained during superfusions up to 4 min; higher concentrations (60–200 nM) evoked inward currents which quickly declined during a 2 min application period (Fig. 1*A*). Repeated superfusions at 10 min intervals with SP at 20 nM produced reproducible,

near-maximal, and maintained responses as illustrated in Fig. 1*A*; therefore, this concentration was used in all experiments with SP described below. Similar results were obtained with VIP.

In contrast to the tachypylaxis observed with concentrations of SP > 30 nM, superfusion at 10 to 15 min intervals with muscarine (1–50 μM), 5-HT (0·1–10 μM) and/or forskolin (0·1–10 μM) evoked inward currents that were reproducible and maintained for up to a 5 min superfusion period (Fig. 2).

Agonist potency. Figure 1*B* plots the concentration–response relations recorded from submucosal neurones in response to superfusion with SP, VIP, 5-HT, muscarine and forskolin. Maximum current amplitude in response to SP (212 ± 85 pA, $n = 60$) was not significantly different from current evoked by forskolin (225 ± 45 pA, $n = 29$; the 5-HT-induced inward current (285 ± 42 pA, $n = 12$) was significantly greater, and both the muscarine (118 ± 19 pA, $n = 14$) and VIP-evoked current (112 ± 22 pA, $n = 8$) were significantly less, than the SP and forskolin responses. Half-maximal concentrations of SP, VIP, forskolin, 5-HT and muscarine were 6, 25, 325, 720 and 2000 nM respectively (Fig. 1*B*).

The actions of dibutyryl cyclic AMP (2 mM, $n = 5$) and 3-isobutyl-1-methylxanthine (IBMX, 0·5 mM, $n = 6$) were identical to forskolin. Similar results have been reported in detail previously for enteric myenteric and submucosal neurones (Nemeth *et al.* 1986; Palmer *et al.* 1986; Mihara *et al.* 1987; Akasu & Tokimasa, 1989).

Occlusion of the slow EPSC and current evoked by SP, 5-HT, muscarine and forskolin

The inward current evoked by SP, 5-HT, muscarine, and forskolin occluded one another and each of these substances also occluded the slow EPSC. Figure 2 illustrates one such experiment in which pressure application of SP evoked no additional inward current when it was applied during superfusion with maximal concentrations of forskolin (Fig. 2*A*), muscarine (Fig. 2*B*) and 5-HT (Fig. 2*C*). In the same neurone, the slow EPSC was not observed in the presence of forskolin (Fig. 2*D*).

SP and 5-HT. Pressure application of SP and superfusion with 5-HT (2 or 6 μM) evoked inward currents of 254 ± 86 and 313 ± 78 pA ($n = 6$); the SP-evoked current was only 60 ± 27 pA (21 % of control) in the presence of 5-HT.

SP and forskolin. In seven neurones, pressure application of SP and superfusion with forskolin (2–6 μM) evoked currents of 195 ± 19 and 161 ± 26 pA respectively; in the presence of forskolin, SP induced a current of 46 ± 11 pA (29 % of control current).

SP and muscarine. Similar results were obtained with SP and muscarine-induced currents: SP current 180 ± 36 pA, muscarine current 100 ± 29 pA ($n = 6$), SP current in the presence of muscarine 45 ± 16 pA (28 % of control).

Other combinations. Qualitatively similar results were obtained when any two agonists were compared, i.e. agonist currents were not additive ($n = 2$ for each combination, i.e. 5-HT *vs*. forskolin, muscarine and the slow EPSC, muscarine *vs*. forskolin and the slow EPSC).

Current–voltage relations

Linear current–voltage relations

In about 14% (9 of 63) of submucosal neurones examined, the steady-state current–voltage (*I–V*) curves were linear between −50 and −120 mV (Fig. 3*A*).

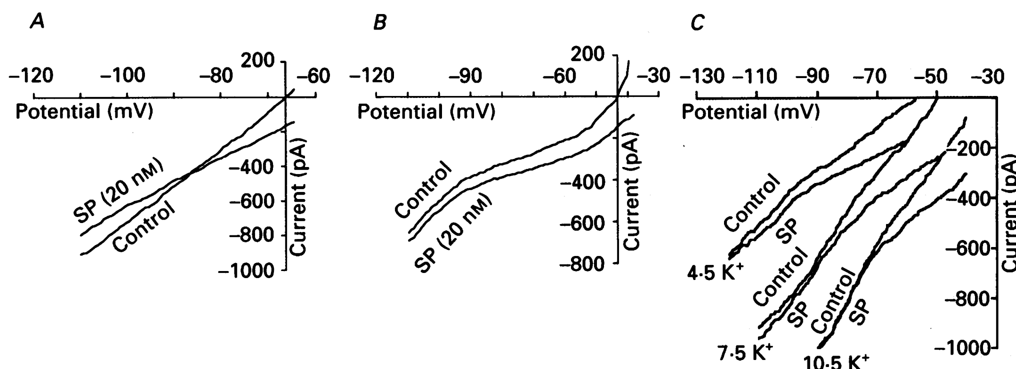

Fig. 3. Linear (*A*) and non-linear (*B* and *C*) *I–V* curves recorded from submucosal neurones in control solution and in the presence of 20 nM SP (as indicated). *A*, SP evoked an inward current which reversed at approximately E_K in those neurones in which a linear *I–V* relation between −60 and −120 mV was observed; this type of response was recorded in a minority (14%) of cells and is not described further herein. *B*, non-linear *I–V* relation typical of those recorded from the majority (86%) of neurones; increased slope conductance is apparent at potential negative to E_K (approximately −90 mV) and positive to −65 mV in both control and SP. SP produced an inward current over the entire voltage range. *C*, recordings obtained from another neurone in which SP evoked an inward current with decreased slope conductance positive to E_K in three different external potassium concentrations (in mM) but no reversal of the current occurred even in high (10·5 mM) external potassium solution.

Substance P, muscarine, 5-HT, and forskolin (n = 3–7) all evoked an inward current at the resting potential associated with a large decrease in the slope conductance over the entire voltage range examined (i.e. −50 to −110 mV; Fig. 3*A*). The SP-induced current reversed polarity at approximately the reversal potential for potassium (E_K; $-93 \pm 1{\cdot}4$ mV, $n = 7$ in 4·5 mM external potassium). Similar results have been described previously for the actions of SP and 5-HT in these neurones (Surprenant *et al.* 1987; Surprenant & Crist, 1988; Akasu & Tokimasa, 1989). The slow EPSC also showed a clear reversal from inward to outward current at the potassium equilibrium potential (approximately −95 mV). Neurones showing this type of response were not studied further.

Non-linear current–voltage relations

The steady-state *I–V* relation of the majority of submucosal neurones has been described previously (Surprenant & North, 1988; Akasu & Tokimasa, 1989); there is a prominent inward rectification at potentials negative to about −85 mV which is due to activation of a barium-sensitive inwardly rectifying potassium conductance

($g_{K,IR}$) and a marked outward rectification at potentials positive to about -60 mV which is due to a calcium-activated potassium conductance ($g_{K,Ca}$; Figs 3*B* and 4*A*). It should be noted that this $g_{K,Ca}$ conductance is prominent in both 'S'-type neurones (this study) as well as 'AH'-type neurones in the guinea-pig submucosal plexus (Akasu & Tokimasa, 1989).

Agonist actions on inwardly rectifying potassium conductance. Substance P ($n = 48$), muscarine ($n = 14$), VIP ($n = 4$), 5-HT ($n = 9$) and forskolin ($n = 17$) all produced an inward current associated with a large decrease in membrane conductance at potentials between -40 and -80 mV; with further hyperpolarization the conductance change became much less apparent and no reversal from inward to outward current was observed at potentials as negative as -130 mV (Fig. 3*B* and *C*). Increasing external potassium from 4·5 to 10·5 mM resulted in agonist-induced currents exhibiting decreased conductance at potentials positive to E_K but still no reversal of the current at potentials negative to E_K ($n = 7$; Fig. 3*C*). Caesium (2 mM), which blocks the $g_{K,IR}$ in a voltage-dependent manner (Surprenant & North, 1988; see also Hagiwara & Takahashi, 1974), did not alter the agonist-induced (SP, 5-HT or forskolin) current over the voltage range of -40 to -110 mV ($n = 4$). Barium (50–100 μM) also prevented the inward rectification (Surprenant & North, 1988) but did not alter the *I–V* relation measured in the presence of SP or forskolin ($n = 5$). These three findings provide good evidence that the slow excitatory transmitters do not alter the resting $g_{K,IR}$ of the membrane (see also Akasu & Tokimasa, 1989).

The calcium-activated potassium conductance. The $g_{K,Ca}$ in these neurones is characterized by a slowly developing inward current in response to hyperpolarizing voltage commands from a holding potential near rest to about -80 mV (Fig. 4*A*); this inward relaxation is due to deactivation of the outward potassium current (see Akasu & Tokimasa, 1989). The potassium current reactivated to produce a slow outward relaxation at the termination of the current pulse (Fig. 4*A*). As expected, superfusion with a low calcium (0·5 mM) solution containing cadmium (100–300 μM) or cobalt (2 mM) abolished the time-dependent current evoked between -40 and -80 mV (Fig. 4*A*). Like the slow excitatory agonists, the low calcium–cadmium solution also produced an inward current at the resting potential (219 ± 13 pA at -50 mV, $n = 11$) with an associated membrane conductance decrease (Fig. 4*B* and *C*).

Cation conductance increase. Substance P ($n = 14$), forskolin ($n = 10$), muscarine ($n = 4$) or 5-HT ($n = 3$) produced a further inward current when applied in the presence of the low calcium–cobalt-containing solution (Fig. 4*B*). The inward current was associated with a small increase in the membrane conductance over the voltage range examined (-40 to -120 mV, Fig. 4*C*). The SP- or forskolin-induced current recorded in the low calcium–cadmium solution was unaltered during 30 min superfusion with the chloride channel inhibitor 9-AC (100 μM, $n = 3$) or by replacing external NaCl with sodium gluconate ($n = 2$). The amplitude of the inward current produced by SP, forskolin or 5-HT was reduced by lowering the external sodium concentration. The peak amplitude produced by SP (in low calcium–cadmium) was 195 ± 18 pA at -50 ($n = 5$); the current was 117 ± 10 pA when the external sodium was halved. No reversal of the current was observed at hyperpolarized (to -130 mV) potentials even in low sodium solution ($n = 5$).

Actions of slow excitatory agonists on potassium conductance increased by somatostatin and α_2-adrenoceptor agonists

Actions of pertussis toxin. Somatostatin and the α_2-adrenoceptor agonist UK 14304 hyperpolarized all neurones depolarized by SP, forskolin, muscarine and 5-HT ($n >$

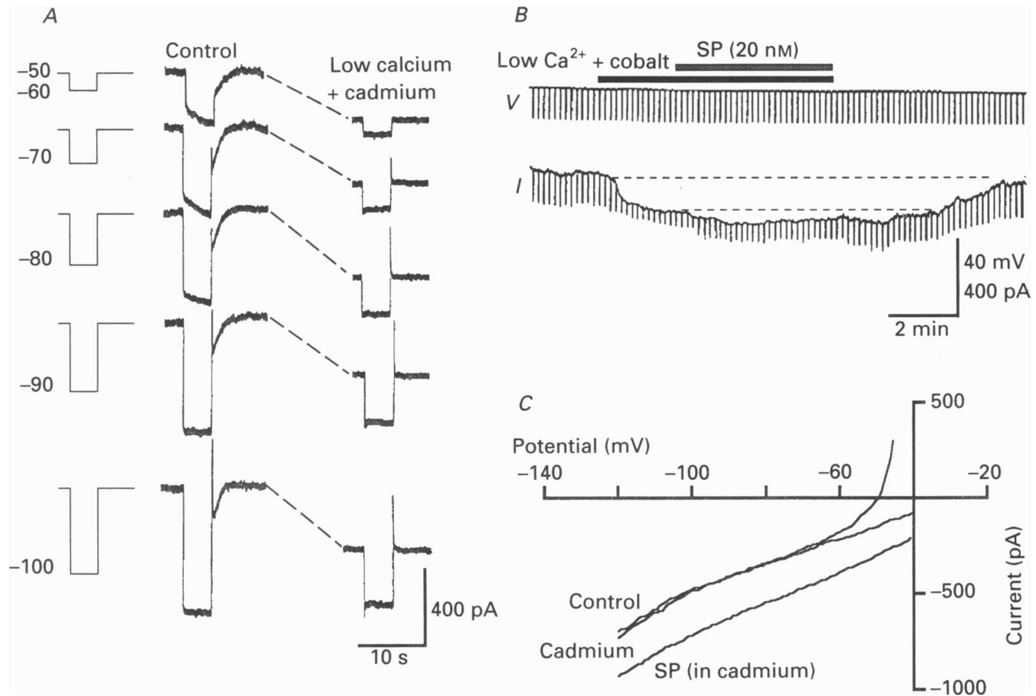

Fig. 4. Actions of low calcium (0·5 mM) plus cadmium (300 μM, *A*) or cobalt (2 mM, *B*) on membrane currents recorded in submucosal neurones and actions of SP after blockade of $g_{K,Ca}$ (*B* and *C*). *A*, currents in response to hyperpolarizing voltage commands (3 s duration) from a holding potential of −50 mV to the indicated potentials in control (left) and in low calcium–cadmium solution (right). The dashed line represents change in holding current in the presence of low calcium–cadmium; inward relaxations were abolished in this solution. The fast outward current following repolarizing is transient A-current. *B*, actions of SP in a low calcium–cobalt solution; low calcium–cobalt caused an inward shift of the holding current (holding potential −55 mV) with concomitant decreased membrane conductance. Superfusion with SP produced a further inward current which was now associated with a conductance increase. *C*, steady-state *I–V* relation recorded from one neurone in control, during superfusion with low calcium–cadmium and when SP was applied in the low calcium–cadmium solution. The low calcium–cadmium abolished the outward rectification at potentials positive to −65 mV and SP now produced an inward current with a small increased membrane conductance.

60); stimulation of the nerve supply to these neurones produced the adrenergic IPSP followed by the slow EPSP. Pertussis toxin pretreatment inhibited the somatostatin- or UK 14304-induced hyperpolarization and the IPSP by greater than 90% (see also Mihara *et al.* 1987; Surprenant & North, 1988) but did not alter the SP ($n = 5$), forskolin ($n = 4$), muscarine ($n = 5$), 5-HT ($n = 3$) current, nor the slow EPSP or EPSC ($n = 5$).

The somatostatin and UK 14304 current. Properties of the outward potassium current activated by somatostatin and α_2-adrenoceptor agonists have been described previously (Mihara *et al.* 1987; Surprenant & North, 1988; Shen *et al.* 1992) and are seen in Figs 5–7. At −60 mV, somatostatin (60 nM) and UK 14304 (600 nM)

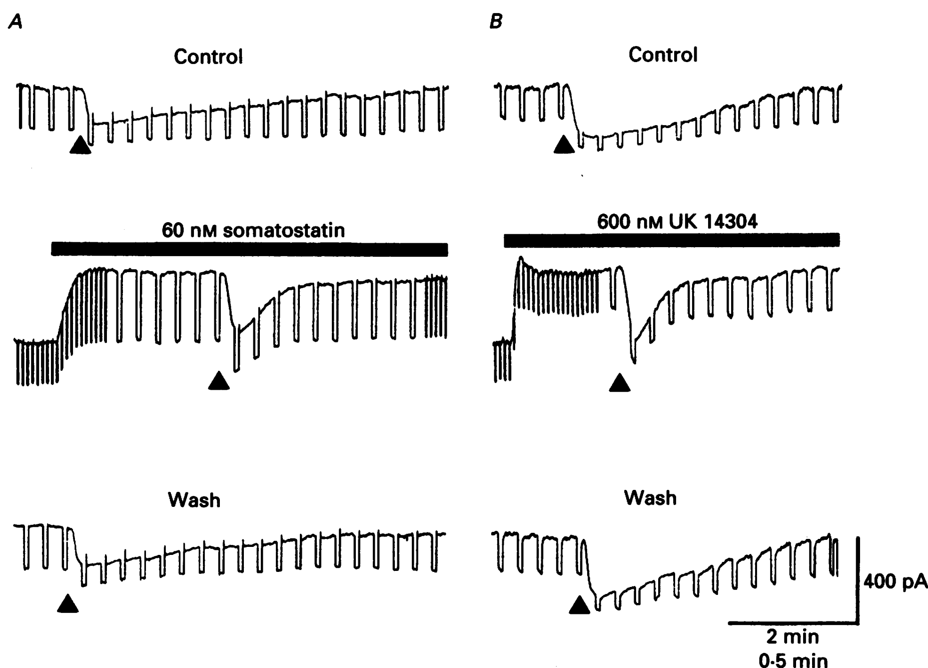

Fig. 5. Inward current to SP was increased in the presence of somatostatin (*A*) or UK 14304 (*B*). Each trace shows pressure-pulse application of SP (at ▲) recorded at the resting potential (−50 mV) in control solution (upper), during the peak outward current produced during superfusion with somatostatin or UK 14304 (middle traces) and approximately 10 min after wash-out. Time calibrations as in Fig. 2.

produced an outward current associated with a significant increase in membrane conductance (Fig. 5); current was 328 ± 54 ($n = 5$) and 317 ± 37 pA ($n = 7$) respectively. The current evoked by either agonist reversed from outward to inward at E_K (Figs 6*B* and 7*B*) and the reversal potential changed in a Nernst fashion with changes in external potassium (Fig. 6*D*). The agonist current showed inward rectification which became more prominent in solutions with increasing potassium concentrations (Fig. 6*D*); this inward rectification was also prevented by barium (30–100 μM, $n = 6$) or caesium (2 mM, $n = 5$).

SP current in the presence of somatostatin or UK 14304. The inward current evoked by brief pressure application of SP at the resting potential was significantly increased in the presence of somatostatin (Fig. 5*A*) or UK 14304 (Fig. 5*B*). The peak inward current to SP was increased by 35 % when it was evoked in the presence of UK 14304 (control amplitude 171 ± 16 pA, amplitude in UK 14304 213 ± 22 pA, $n = 8$). The SP current was increased by 45 ± 21 % in the presence of somatostatin (control

256 ± 90 pA, in somatostatin 294 ± 37 pA). The percentage decrease in membrane conductance produced by SP at the resting potential was 51 ± 5 ($n = 8$) and 49 ± 3 % ($n = 5$) in the presence of UK 14304 and somatostatin, whereas it was 31 ± 5 and 34 ± 2 % in the absence of these two agonists. These results suggest that SP

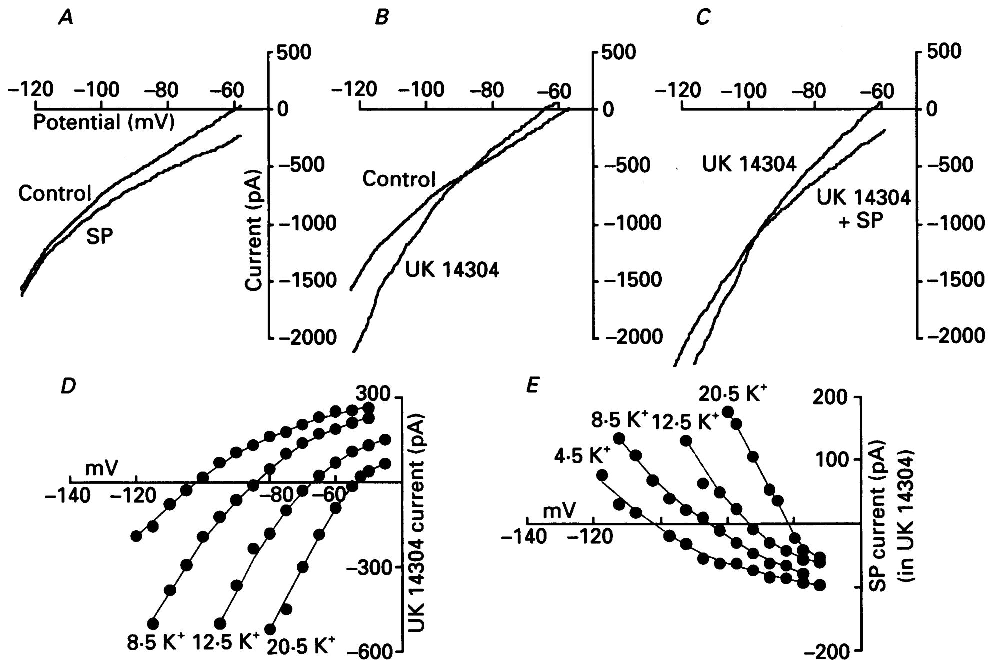

Fig. 6. SP inhibition of the potassium current activated by UK 14304. *A*, *I–V* plots from one neurone in control and during superfusion with SP; no reversal of the SP current was apparent. *B*, *I–V* plot in control (same control curve as that in *A*) and during UK 14304 application; the UK 14034 outward current reversed to inward current at approximately −92 mV. *C*, *I–V* plot in UK 14304 (same trace as that in *B*) and during superfusion with SP which now produced an inward current that reversed to an outward current at about −98 mV. *D*, UK 14304-induced current obtained by subtracting the control current from current in the presence of UK 14304, plotted as a function of membrane potential; the experiment was performed in four different potassium concentrations (in mM, as indicated). Note the inward rectification of the UK 14304-induced currents which increased with increasing potassium concentration. *E*, the SP-induced current in the presence of UK 14304 was determined by subtracting current in UK 14304 plus SP from current in UK 14304 in four potassium concentrations. Results in *D* and *E* obtained from the same neurone; note the reversal potentials of the SP (in UK 14304) currents (*E*) are about 5 mV more negative than the UK 14304 currents (*D*).

can decrease the potassium conductance activated by the 'inhibitory' agonists somatostatin and UK 14304.

The apparent inhibition of the α_2-adrenoceptor and somatostatin-activated potassium conductance was studied further by measuring *I–V* curves produced by SP and other slow excitatory agonists in the absence and presence of UK 14304. As described above, SP produced an inward current and decreased slope conductance

from −60 to −90 mV but no reversal of the current was apparent (Fig. 6*A*). UK 14304 produced an outward current which reversed to an inward current at −92 mV (Fig. 6*B*). When SP was applied in the presence of UK 14304, the *I–V* curve again showed the inward current with decreased conductance between −60 and −90 mV but now the current reversed to an outward current at −98 mV (Fig. 6*C*). The reversal potential of the SP or forskolin current in the presence of UK 14304 also shifted with the extracellular potassium concentration in an approximately Nernst fashion (Fig. 6*D* and *E*). The SP reversal potential in the presence of UK 14304 was 5–8 mV more negative than the UK 14304 reversal potential measured in the same cell ($n = 5$, compare Fig. 6*D* and *E*).

The decrease in membrane conductance to SP was $2{\cdot}0 \pm 0{\cdot}7$ and $-0{\cdot}01 \pm 0{\cdot}7$ nS at −60 and −110 mV respectively ($n = 7$); in the same neurones UK 14304 caused an increase in slope conductance of $4{\cdot}6 \pm 2{\cdot}1$ and $25{\cdot}6 \pm 4$ nS at the same potentials. SP decreased the *increased* slope conductance produced by UK 14304 to $4{\cdot}0 \pm 1{\cdot}5$ and $12{\cdot}0 \pm 1{\cdot}8$ nS at −60 and −110 mV. Similar results were obtained with muscarine, forskolin and 5-HT ($n = 2$).

Figure 7*D–E* shows results obtained when these experiments were carried out in low calcium–cadmium solution to abolish $g_{K,Ca}$ and its contribution to the agonist-evoked currents. In this experiment, forskolin produced an inward current with an increased slope conductance from −50 to −130 mV and no evidence of reversal at very hyperpolarized potentials (Fig. 7*D*); UK 14304 produced its usual effect, an increase in an inwardly rectifying potassium conductance with a reversal at E_K (Fig. 7*E*). Forskolin, applied in the presence of UK 14304, produced an inward current with a slightly decreased slope conductance and a negative reversal potential (−130 mV, Fig. 7*F*). Similar results were obtained in four additional experiments with SP or muscarine.

We have not examined possible actions of the slow excitatory agonists on other currents known to be present in enteric neurones, specifically delayed rectifier potassium current, A-type potassium current, or hyperpolarization-activated cation current (H-current). Akasu & Tokimasa (1989) have shown that SP does not affect A-current in submucosal neurones; H-current is present only rarely in submucosal neurones (Galligan, Tatsumi, Shen, Surprenant & North, 1990) and neurones possessing H-current were not examined in this study.

Comparison of measured and simulated I–V relations

We computed the steady-state *I–V* relation from −50 to −130 mV for a cell in which five conductances were operative: a resting or leak g_K ($g_{K,L}$), an inward rectifier g_K ($g_{K,IR}$), a $g_{K,Ca}$, an agonist-activated inwardly rectifying g_K ($g_{K,agonist}$), and an agonist-activated non-selective cation conductance (g_{cation}) (see Methods) and compared simulations with *I–V* curves measured in control or low calcium–cadmium solution, in the presence of SP, forskolin or muscarine, in the presence of UK 14304 and in the presence of slow excitatory agonists plus UK 14304. Results obtained from two neurones are shown in Fig. 7 in which dotted lines represent computed *I–V* curves and continuous lines are recorded currents. In the experiments shown in Fig. 7*A–C*, the control *I–V* curve was well fitted by the sum of $g_{K,L}$, $g_{K,Ca}$, and $g_{K,IR}$ and the muscarine current was well fitted by assuming muscarine reduced $g_{K,Ca}$ by 95% in addition to activating a g_{cation} of 0·5 nS (Fig. 7*A*); the *I–V* curve in UK 14304 alone produced a good fit by assuming that only $g_{K,agonist}$ was activated (Fig. 7*B*).

The best fit to the I–V curve in the presence of muscarine plus UK 14304 was obtained by assuming muscarine depressed $g_{K,agonist}$ by 60% in addition to its actions on $g_{K,Ca}$ and g_{cation} (Fig. 7*C*). We made similar computations based on recordings obtained in a further four experiments; in each case the best fit to the SP,

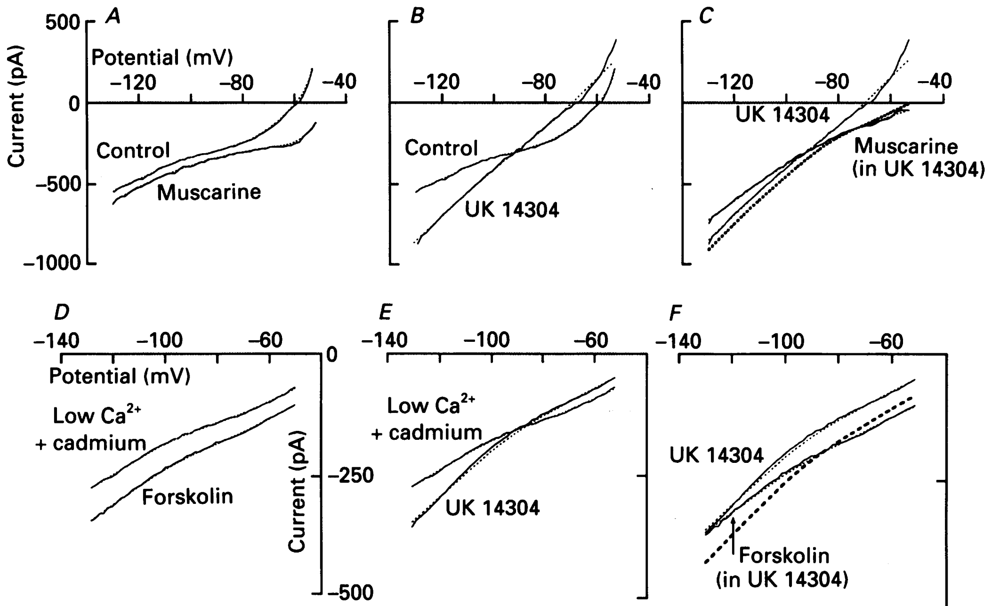

Fig. 7. I–V curves recorded (continuous lines) and computed (dotted lines) using functions described in Methods. *A*, the computed curve in control solution was obtained by using values for $g_{K,L}$ at -60 mV of 1 nS, for $g_{K,Ca}$ at -60 mV of 6 nS and maximum $g_{K,IR}$ of 4 ns (for E_K of -90 mV). Muscarine-induced current was computed by using a value of 0·25 nS for $g_{K,Ca}$ (i.e. 95% inhibition of $g_{K,Ca}$) and adding a g_{cation} value of 0·5 nS with values for $g_{K,L}$ and $g_{K,IR}$ remaining constant. *B*, control curve is same as *A*. UK 14304 curve computed by adding a $g_{K,agonist}$ value of 3 nS (at -60 mV). *C*, UK 14304 curve same as in *B*. Simulated current in the presence of muscarine plus UK 14304 obtained by assuming muscarine evoked same response as in *A* and additionally decreased $g_{K,agonist}$ by 60%. The heavy dotted line in *C* represents the I–V curve expected if muscarine did not alter $g_{K,agonist}$. Recordings in *A*–*C* from the same neurone; muscarine concentration was 6 μM. *D*–*F*, I–V curves recorded from another neurone in a low calcium–cadmium solution throughout. *D*, simulated curve in low calcium–cadmium obtained using $g_{K,L}$ 3 nS, $g_{K,Ca}$ 0, $g_{K,IR}$ 4 nS, that in forskolin by simply adding a g_{cation} of 1·1 nS. *E*, simulated curve in UK 14304 produced by adding $g_{K,agonist}$ of 1·6 nS to the low calcium–cadmium curve. *F*, the I–V curve recorded in the presence of forskolin and UK 14304 was best fitted by assuming forskolin inhibited the UK 14304 current by 90% in addition to activating $g_{K,cation}$ of 1 nS. Heavy dashed line in *F* is the expected curve if forskolin did not alter $g_{K,agonist}$.

muscarine or forskolin I–V curve was obtained by assuming these agonists did not alter $g_{K,L}$ or $g_{K,IR}$ but decreased $g_{K,Ca}$ (by 35–95%) and activated g_{cation} (of 0·1 to 1 nS). I–V curves obtained in the presence of UK 14304 and SP (or forskolin) were also best fitted by a model that incorporated a decrease in $g_{K,agonist}$ (by 40–90%) in addition to the actions on $g_{K,Ca}$ and g_{cation}.

DISCUSSION

Results obtained in the present study confirm and significantly extend previous investigations of mechanisms by which slow excitatory transmitters modulate ion conductances in enteric neurones. In confirmation of previous results with SP (Akasu & Tokimasa, 1989), we found the primary means by which SP, as well as muscarine, 5-HT, VIP and the slow excitatory transmitter(s) released from nerves depolarize submucosal neurones is by inhibiting the slow $g_{K,Ca}$. In addition, we found these agonists activated a cation conductance and decreased both a background ('leak') and an inwardly rectifying potassium conductance that is activated by the inhibitory agonists, somatostatin and noradrenaline. Finally, we conclude that a common initial event underlies the modulation of all of these conductances by each of these agonists: stimulation of adenylate cyclase.

Inhibition of slow $g_{K,Ca}$

Three distinct calcium-activated or calcium-dependent potassium conductances present in innumerable neurones have been shown repeatedly to be the target of slow excitatory transmitters such as SP and muscarine; these are the M-current, the after-hyperpolarization (AHP) current and the slow $g_{K,Ca}$ current described in the present study (reviewed by Tokimasa & Akasu, 1992). The slow $g_{K,Ca}$ in enteric neurones is certainly the most distinguishing feature of these mammalian autonomic neurones as it rarely has been described in other neurones; its extremely slow kinetics (Fig. 4*A*, see Morita, North & Tokimasa, 1982; North & Tokimasa, 1983, 1987; Akasu & Tokimasa, 1989) clearly separate it from the M-current whose pharmacology and voltage-dependence it otherwise resembles (Adams, Brown & Constanti, 1982*a*, *b*; Brown, 1988; Tokimasa & Akasu, 1992). Its insensitivity to both curare and apamin differentiate it from the AHP current found in numerous peripheral and central neurones (e.g. Pennefather, Lancaster, Adams & Nicoll, 1985; Schwindt, Spain, Foehring, Chubb & Crill, 1988). The M-current in other autonomic neurones and the slow $g_{K,Ca}$ in enteric neurones, but not the AHP current, contribute substantially to the resting conductance of the cell; measurements based on changes in conductance (at rest) that occur on application of maximum concentrations of muscarine and SP or on removal of external calcium show that 20–50% of total membrane conductance may be due to M-current in sympathetic ganglia or the slow $g_{K,Ca}$ in enteric neurones (e.g. Fig. 4*A*; North & Tokimasa, 1987; Brown, 1988; Akasu & Tokimasa, 1989; Tokimasa & Akasu, 1992). The presence in most peripheral neurones of one or the other of these potassium currents and the realization that they appear to be the prime targets for modulation by slow excitatory neurotransmitters emphasize their important physiological role in synaptic transmission throughout the autonomic nervous system.

Inhibition of background potassium conductance

Muscarine, SP, 5-HT and/or VIP also inhibit a background (or 'leak') potassium conductance in autonomic and central neurones (see Tokimasa & Akasu, 1992, for review on autonomic neurones; see Nicoll, Malenka & Kauer, 1989, for review on

central neurones). In most instances, this potassium conductance is less affected by agonists than is the M-current, slow $g_{K,Ca}$ or AHP current (e.g. hippocampal neurones (Nicoll *et al.* 1989); amphibian sympathetic neurones (Tokimasa & Akasu, 1992); enteric submucosal neurones (this study and Akasu & Tokimasa, 1989)). In the majority of neurones examined in the present study, the contribution that inhibition of this leak conductance, $g_{K,L}$ made to the overall conductance change underlying the slow EPSC or agonist-induced current was negligible in comparison to the contribution made by $g_{K,Ca}$ and g_{cation}. However, it is most likely that $g_{K,L}$ is inhibited to some degree in all neurones; our simulations (e.g. Fig. 7) showed that a 5–10% inhibition of this conductance, in addition to the effects of $g_{K,Ca}$ and g_{cation}, could adequately describe the I–V relations recorded from most neurones.

Activation of cation conductance

The conclusion that SP and other slow excitatory agonists increased a cation conductance in submucosal neurones is based most strongly on results obtained under conditions where $g_{K,Ca}$ was absent (i.e. in low calcium–cadmium solution). In this environment, SP, muscarine, 5-HT and forskolin all produced an inward current associated with an increased slope conductance over the voltage range studied (-50 to -130 mV). The inward current was unaltered by the chloride channel blocker 9-AC or by changing external chloride concentrations but was decreased in low sodium solutions. The inclusion of a cationic conductance activated by these agonists also was required in our simulations in order to produce a good fit to the measured I–V curves in both control and low calcium–cadmium solution (Fig. 7). An increased cation conductance produced by VIP has been noted previously in enteric neurones (Mihara *et al.* 1986); however, it often has been noted that the slow EPSC, the SP- (Surprenant, 1984, 1989) and the 5-HT- (Surprenant & Crist, 1988) induced currents in submucosal neurones fail to reverse or reverse at a membrane potential significantly more negative than E_K. In previous studies, it was suggested this may result from agonist inhibition of voltage-independent background potassium channels located at an electronically distant site from the cell body recording site. It now seems clear that the contribution from the cation conductance increase is the more likely explanation for the non-reversing I–V curves.

Muscarine has long been known to increase a cation conductance in amphibian sympathetic ganglion cells (Kuba & Koketsu, 1977; Tsuji & Kuba, 1988), as well as in cardiac and smooth muscle (Benham, Bolton & Lang, 1985; Inoue, Kitamura & Kuriyama, 1987; Matsumoto & Pappano, 1989). More recently, muscarine has been shown to similarly increase a cation conductance in central neurones (rat locus coeruleus, Shen & North, 1992). In all of these preparations, as in submucosal neurones, slow excitatory agonists also decrease a potassium conductance. Thus, a dual cation conductance increase–potassium conductance decrease appears to be the rule rather than the exception; the contribution one or the other makes to the response recorded at rest appears to depend largely on the potassium conductance available to be acted upon. For example, an inwardly rectifying potassium conductance ($g_{K,IR}$) is decreased by muscarine and SP in locus coeruleus neurones; this potassium conductance contributes little to the resting conductance and consequently the major component of the excitation is due to the cation conductance

increase (Shen & North, 1992). In submucosal neurones, the major component of slow excitation at rest is due to the $g_{K,Ca}$ decrease; only at hyperpolarized levels does the cation conductance become prominent.

Inhibition of potassium conductance activated by somatostatin and UK 14304

Our experiments provide good evidence that SP, muscarine, 5-HT and forskolin inhibit the potassium current activated by agonists which inhibit neuronal excitability in these cells. In the presence of the 'inhibitory' agonists, the SP-induced inward current was increased, as would be expected if an additional set of potassium channels were now available to be closed by the slow excitatory agonists. SP, muscarine and forskolin evoked inward currents in control solution which did not show reversals at hyperpolarized levels (due to the cation conductance increase, see above) but $I-V$ curves constructed in the presence of one of the slow excitatory agonists plus somatostatin or UK 14304 reversed from inward to outward at potentials slightly negative to E_K. Our simulations indicated that this shift in the SP-evoked $I-V$ curves in the presence of somatostatin or UK 14304 would be expected only if SP and the other excitatory agonists decreased the potassium current ($g_{K,agonist}$) activated by the inhibitory agonists. Surprisingly, the converse result was not obtained; that is, the outward current produced by noradrenaline or somatostatin does not appear to be significantly decreased when these agonists are applied in the presence of forskolin (Mihara *et al.* 1987). It may be that the potassium conductance coupled to these inhibitory agonists must first be activated by agonist in order for forskolin and the other excitatory agonists to be effective.

The inwardly rectifying $g_{K,agonist}$ in submucosal neurones is indistinguishable from the resting inward rectifier ($g_{K,IR}$) in its pharmacology and its dependence on both voltage and potassium concentration (Surprenant & North, 1988). More recently, we also found that both somatostatin and UK 14304 increased the probability of opening of single potassium channels which were already active in an excised (outside-out) patch obtained from submucosal neurones, but the agonists never caused the opening of a potassium channel distinct from those active in the absence of agonist (Shen *et al.* 1992). We supposed that these results could best be explained by assuming that somatostatin and α_2-adrenoceptor agonists shifted the voltage dependence of activation of the resting $g_{K,IR}$ to more positive potentials, thus accounting for the ability of these agonists to produce outward currents at potentials positive to E_K. Results obtained in the present study appear to contradict such an interpretation because SP, muscarine and forskolin inhibited $g_{K,agonist}$ but did not inhibit $g_{K,IR}$. Further studies to adequately identify the potassium channels underlying both $g_{K,agonist}$ and $g_{K,IR}$ will be required before the above interpretation can be ruled out, or another interpretation devised. Nevertheless, this study has provided clear demonstration that slow excitatory agonists are able to inhibit the same potassium conductance activated by inhibitory agonists in a single neurone.

Intracellular transduction pathway

Many previous studies in enteric neurones have concluded that the potassium conductance decrease produced by SP, muscarine, 5-HT, VIP and other slow excitatory agonists is due to stimulation of the adenylate cyclase pathway (Nemeth

et al. 1986; Palmer *et al.* 1986; Mihara *et al.* 1987; Akasu & Tokimasa, 1989). Our results provide further corroboration for this conclusion, in particular the mimicry of the slow excitatory response by cholera toxin, which is well characterized to catalyse the ADP ribosylation of the stimulatory guanine nucleotide binding protein of the adenylyl cyclase system ($G_{s\alpha}$, Moss & Vaughan, 1988). Activation of $G_{s\alpha}$ universally leads to stimulation of the adenylate cyclase–cyclic AMP pathway (Gilman, 1987); thus, cholera toxin would be expected to mimic the actions of SP and muscarine if, indeed, this pathway mediates their effects. Our results further indicate that stimulation of adenylate cyclase can account for all the actions of the slow excitatory agonists: the decreased $g_{K,Ca}$, the decreased background conductance ($g_{K,L}$) observed in the minority of cells (see Akasu & Tokimasa, 1989), the decreased $g_{K,agonist}$ as well as the increased cation conductance. We base this conclusion on the findings that forskolin, IBMX, dibutyryl cyclic AMP and cholera toxin altered all of these ionic conductances in a manner identical to that produced by SP, muscarine, 5-HT, VIP, or the slow EPSC in the same neurone. We conclude that in submucosal neurones there is convergence of many receptors onto a single intracellular site of action which, in turn, diverges onto multiple ion channels to produce neuronal excitation.

This work was supported by US Department of Health and Human Services grant NS 25996.

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