Contribution of chloride conductance increase to slow EPSC and tachykinin current in guinea-pig myenteric neurones

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1. Single electrode voltage clamp recordings were obtained from myenteric neurones of guinea-pig ileum *in vitro*. Slow excitatory postsynaptic currents (sEPSCs) were elicited by focal stimulation of interganglionic nerve strands in twenty-four of thirty neurones more than 30 min after impalement. In seventeen of twenty-four neurones, sEPSCs were associated with a conductance decrease and reversed polarity at -96 ± 3 mV (near the reversal potential for potassium, E_K); this response was due to inhibition of resting potassium conductance, g_K. In seven of twenty-four neurones, there was either no net conductance change or a biphasic conductance change during the sEPSC; a reversal potential for peak currents could not be determined.

2. Application of senktide (3 μM), a neurokinin-3 receptor agonist, caused an inward current in forty-one of fifty-three neurones more than 30 min after impalement. In twenty of forty-one neurones, senktide-induced currents were due to inhibition of resting g_K. In eleven of forty-one neurones there was either no net conductance change or a biphasic conductance change; a reversal potential for peak currents could not be determined. In ten out of forty-one neurones, senktide-induced currents were associated with a conductance increase (g_{inc}); the estimated reversal potential was -17 ± 3 mV.

3. Application of forskolin (1 μM) caused an inward current that occluded the decrease in g_K caused by senktide and the sEPSC. In neurones in which sESPCs and senktide responses were associated with an unclear or biphasic conductance change, forskolin did not reduce the peak current and residual currents were usually associated with a g_{inc}.

4. In neurones in which senktide-induced currents were associated with a g_{inc}, reducing extracellular Cl^- to 13 mM reduced senktide-induced currents by 79%. Reducing extracellular Na^+, or adding tetraethylammonium (TEA, 50 mM), cobalt (2 mM) or picrotoxin (30 μM) did not change senktide-induced currents. The chloride transport/channel blockers niflumic acid and mefenamic acid (both at 100 μM) blocked senktide-induced currents. It was concluded that senktide increases chloride conductance (g_{Cl}).

5. Chord conductance measurements made between -70 and -90 mV during sEPSCs were used to determine the contribution of an increase in g_{Cl} to sEPSCs. These measurements indicated that the peak sEPSC is composed of a 90% decrease in g_K and a 10% increase in g_{Cl}. Similar data were obtained from measurements made during senktide responses. It was concluded that senktide-induced currents and sEPSCs are due to a concurrent decrease in g_K and an increase in g_{Cl} in a subset of myenteric neurones.

Excitatory synaptic transmission in the enteric nervous system can be placed into two categories: fast ligand-gated transmission and slow transmission. Fast excitatory postsynaptic potentials (fEPSPs) are the major form of synaptic transmission between enteric neurones, and are mediated primarily by acetylcholine acting at nicotinic receptors. Single electrical stimuli applied to presynaptic nerve fibres cause a fEPSP that is due to an increase in non-specific cation conductance (Nishi & North, 1973; Hirst, Holman & Spence, 1974; Wood, 1989). Slow excitatory postsynaptic potentials (sEPSPs) are caused by trains of electrical stimuli applied to presynaptic nerve fibres. The sEPSP is a long-lasting membrane depolarization that is usually associated with an increase in membrane input resistance (Johnson, Katayama & North, 1980; Grafe, Mayer & Wood, 1980). The increase in input resistance is caused by transmitter-induced inhibition of two types of resting potassium conductance

(g_K). The first conductance is a background g_K present in all enteric neurones. The second conductance is a calcium-activated potassium conductance ($g_{K,Ca}$) that is present mainly in after-hyperpolarization (AH)-type neurones (Morita, North & Tokimasa, 1982; North & Tokimasa, 1987; Galligan, Tokimasa & North, 1987). $g_{K,Ca}$ contributes to the resting membrane potential and also gives rise to the long-lasting spike after-hyperpolarization in AH neurones. The after-hyperpolarization is due to activation of $g_{K,Ca}$ by calcium entering the neurone during an action potential (Nishi & North, 1973; Hirst *et al.* 1974). During the sEPSP, resting and action potential-activated $g_{K,Ca}$ are inhibited (Grafe *et al.* 1980). While most sEPSPs are due to inhibition of resting g_K, some sEPSPs in enteric neurones are associated with a decrease in input resistance or no change in input resistance. In neurones in which there is a decrease in input resistance the reversal potential for the sEPSP is near −10 mV (Mawe, 1990). In neurones in which there is no apparent change in input resistance an estimate of the reversal potential was not possible (Shen & Suprenant, 1993).

Many putative neurotransmitters including substance P (SP), 5-hydroxytryptamine (5-HT), vasoactive intestinal polypeptide, gastrin releasing peptide, and calcitonin gene-related peptide mimic the sEPSP when applied to enteric neurones (Wood & Mayer, 1978; Katayama, North & Williams, 1979; Zafirov, Palmer, Nemeth & Wood, 1985; Palmer, Wood & Zafirov, 1987). The intracellular transduction mechanism for mediators of the sEPSP may involve a cAMP–protein kinase A-dependent pathway because forskolin, an activator of adenylate cyclase (Seamon & Daly, 1986), mimics the sEPSP (Nemeth, Palmer, Wood & Zafirov, 1986). A diacylglycerol–protein kinase C-dependent pathway may also contribute to sEPSPs in some enteric neurones (North, Williams, Surprenant & Christie, 1987; Bertrand & Galligan, 1993*a*). It is likely that several neurotransmitters as well as multiple intracellular transduction pathways are involved in the generation of sEPSPs.

Senktide (succinyl-[Asp^6,*N*-Me-Phe^8]-SP(6–11)) is a selective neurokinin-3 (NK-3) receptor agonist (Hanani, Chorev, Gilon & Selinger, 1988). The NK-3 receptor is believed to be the neuronal tachykinin receptor (Guard, Watson, Maggio, Phon Too & Watling, 1990). Both SP and senktide mimic the sEPSP because NK-3-mediated responses are associated with a membrane depolarization, an increase in resistance, an inhibition of resting g_K and inhibition of spike-activated $g_{K,Ca}$ (Katayama & North, 1979; Hanani *et al.* 1988; Morita & Katayama, 1992). However, other studies have shown either no resistance change or resistance decreases during depolarizations induced by tachykinin peptides (Galligan *et al.* 1987). This phenomenon has been ascribed to membrane rectification during uncontrolled depolarizations (Katayama *et al.* 1979; Hanani & Burnstock, 1985) or actions of transmitter at sites electrically distant from the recording electrode (Surprenant, 1984). However, recently it has been shown that in submucosal ganglia, SP, muscarine and 5-HT caused a simultaneous increase in a cation conductance and a decrease in g_K (Shen & Surprenant, 1993). It was suggested that two conductance changes could mediate the sEPSP in some submucosal neurones (Shen & Surprenant, 1993).

The purpose of this study was to use single electrode voltage clamp to examine in detail conductance changes associated with senktide-induced currents and slow excitatory postsynaptic currents (sEPSCs) in myenteric neurones *in vitro*. We provide evidence that senktide and the mediators of the sEPSC simultaneously inactivate a potassium conductance and activate a chloride conductance. Some of these data have been published previously in abstract form (Bertrand & Galligan, 1993*a*, *b*).

METHODS

Tissue preparation

Guinea-pigs (male 250–350 g, obtained from the Michigan Department of Public Health, Lansing, MI, USA) were anaesthetized with 100% halothane, stunned and bled from the neck. A 5–6 cm segment of ileum, taken 10–20 cm from the ileocaecal junction was removed and placed in oxygenated (95% O_2–5% CO_2) Krebs solution of the following composition (mM): NaCl, 117; NaH_2PO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 2·5; KCl, 4·7; $NaHCO_3$, 25; and glucose, 11. The Krebs solution also contained nifedipine (1 μM) and scopolamine (1 μM) to reduce movements of the longitudinal muscle during intracellular recordings. The segment of ileum was cut open along the mesenteric attachment and pinned mucosal side up in a silastic elastomer-lined Petri dish. The mucosa, submucosa and circular muscle were removed, leaving the myenteric plexus with attached longitudinal muscle. This preparation was then transferred to the base of a small silastic elastomer-lined recording chamber (volume < 2 ml), stretched, pinned flat, and superfused with warmed Krebs solution (34–36 °C) at a flow rate of 3 ml min^{-1}.

Electrophysiology

Myenteric ganglia were visualized at ×200 magnification with differential interference contrast optics and neurones were impaled with glass microelectrodes (80–120 MΩ tip resistance) containing 2 M KCl. Synaptic currents were elicited by focal stimulation (20 Hz, 500 ms train duration, 0·5 ms pulse duration, 40–60 V) of circumferentially orientated interganglionic nerve tracts with a broken-back glass micropipette filled with Krebs solution. Current measurements were made using a single electrode voltage clamp amplifier (Axoclamp 2A; Axon Instruments, Foster City, CA, USA). The switching frequency was 3 kHz and the duty cycle was 70% voltage measuring, 30% current passing. The voltage at the head stage was monitored on a separate oscilloscope to ensure that it had settled to its control level at the time it was sampled. In most experiments the holding potential (V_h) was −70 mV. Voltage steps (V_{step}) of 10–30 mV in amplitude negative to V_h

and 300–500 ms in duration were evoked at approximately 2·2 s intervals at rest and during agonist- or nerve-mediated current responses. Chord conductances were calculated from the current amplitudes measured at the end of the voltage steps or from current–voltage relationships. Current–voltage (I–V) relationships were calculated by measuring the peak amplitude of individual responses at different holding potentials or by measuring the steady-state I–V relationship. Steady-state I–V relationships were measured by generating a series of voltage steps (300 to 500 ms duration) to different test potentials between −40 and −110 mV at rest and during evoked responses. In the case of transient responses (such as sEPSCs and senktide responses) only a few voltage steps could be evoked at peak currents (see Fig. 1), and therefore several consecutive responses were used.

Analysis of conductance and statistics

Chord conductance measurements (see above) were used to determine the contribution of multiple conductance changes to sEPSCs or senktide responses. The observed reversal potential of the conductance increase (−17 mV) suggested that it may be either a chloride conductance or a non-specific cation conductance. In myenteric neurones, the chloride equilibrium potential has been estimated at −18 mV by measuring the reversal potential of $GABA_A$-activated chloride responses (Cherubini & North, 1984; Bertrand & Galligan, 1992). Similarly, the reversal potential for a non-specific cation current in myenteric neurones has been estimated to be between −25 and −10 mV by measuring the reversal potential of the hyperpolarization-activated cation current (I_H) or the current activated by acetylcholine acting at nicotinic receptors (Galligan, Campbell, Kavanaugh, Weber & North, 1989; Galligan, Tatsumi, Shen, Surprenant & North, 1990). The results of the analysis described below will hold true for an increase in chloride conductance or an increase in a non-specific cation conductance.

The predicted current resulting from a decrease in potassium conductance combined with a conductance increase (g_{inc}) is given by:

$$\Delta I = [\Delta g_K (V_h - E_K)] + [\Delta g_{inc} (V_h - E_{inc})], \quad (1)$$

where ΔI is equal to the agonist-induced current, Δg_K is the change in potassium conductance, V_h is the holding potential, E_K is the potassium equilibrium potential (−90 mV), Δg_{inc} is the increase in conductance (for example, chloride or cation), and E_{inc} is the equilibrium potential for the current passing through g_{inc}. The measured conductance change Δg is given by $\Delta g = \Delta g_K + \Delta g_{inc}$, and substitution in eqn (1) leads to:

$$\Delta I = [\Delta g (V_h - E_K)] + [\Delta g_{inc} (E_K - E_{inc})]. \quad (2)$$

This analysis was used to fit I–V relationships with a one- or two-parameter model in order to determine the significance of Δg_{inc}. A least-squares fit of eqn (2) (ΔI as a function of Δg) was used to determine whether the experimental data were fitted best when Δg_{inc} was zero (one parameter) or when Δg_{inc} was allowed to take a value other than zero (two parameter). ΔI and Δg were measured, V_h was known and E_K and E_{inc} were assigned values of −90 and −18 mV, respectively.

For some cells, I–V relationships were not available and the relative contributions of Δg_{inc} and Δg_K were determined from chord conductance determinations made at a single holding potential. These analyses were used to characterize the time course of Δg_K and Δg_{inc} and to select neurones with relevant responses for further pharmacological analysis. In these cases, Δg_K was determined from the calculated value of Δg_{inc} and the observed Δg and ΔI (see Fig. 4).

Data are expressed as means ± standard error of the mean. Differences between mean estimates were tested for significance using Student's paired t test. Reversal potentials and chord conductances were estimated from I–V plots using a least-squares linear regression analysis. Time constants for rise and decay of currents were fitted using a double exponential equation.

Drugs

Drugs were applied by three methods: (1) addition to the superfusing Krebs solution in known concentrations; (2) pressure ejection from a micropipette (10–20 μm tip diameter) positioned within 200 μm of the impaled neurone; or (3) fast flow superfusion of the ganglia using 300 μm inner diameter capillary tubing connected to drug-containing reservoirs. Tetrodotoxin (TTX, 0·3 μM) was added to the superfusing Krebs solution when sEPSCs were not being evoked in order to block Na^+-dependent action potentials and to minimize the influence of synaptically connected neurones. Caesium chloride (2 mM) was added during conductance measurements and during isolation of the senktide-induced conductance increase to block I_H (Galligan *et al.* 1990). All drugs were purchased from Sigma Chemical Company (St Louis, MO, USA).

RESULTS

Characterization of neurones

Data were obtained from S-neurones and AH-neurones (Hirst *et al.* 1974). S-neurones received fast and slow synaptic input, had a linear I–V relationship and did not respond to GABA. AH-neurones received slow synaptic input, had a non-linear I–V relationship and spike after-hyperpolarizations or after-currents of greater than 1 s duration. GABA, applied by pressure produced fast (time to peak < 3 s) inward currents in AH-neurones (Cherubini & North, 1984). Recordings lasting from 0·5 to 3 h were made from 50 S-neurones and 200 AH-neurones in 130 preparations. Extended impalement times, drug application and nerve stimulation were associated with a slowly developing decrease in membrane conductance, a depolarized resting membrane potential and increased neuronal excitability. During the course of an experiment the resting conductance of neuronal membranes decreased from an initial value of 16·1 ± 2·3 to 12·1 ± 1·3 nS more than 30 min after impalement in S-neurones ($n = 15$), and from 25·7 ± 4·2 to 11·9 ± 1·1 nS in AH-neurones over a similar time course ($n = 17$). The decrease in membrane conductance was due to inhibition of resting g_K (Surprenant, North & Katayama, 1987; Wood, 1989). Consequentially, sEPSCs and senktide responses elicited soon after impalement were composed predominantly of a decrease in g_K, while responses elicited later (> 30 min after impalement) were more likely to exhibit an increase in conductance (g_{inc}).

The sEPSC is due to two conductance changes

Stimulation of presynaptic nerves 10–30 min after impalement elicited sEPSCs in 175 of 200 neurones. In 160 of 175 (91%) neurones, the sEPSC was associated with a conductance decrease. In 15 of 175 (9%) neurones, there was either no measurable conductance change or a biphasic conductance change.

An *I–V* relationship was obtained for the sEPSC in twenty-four AH-neurones at least 30 min after impalement. In these neurones, peak currents and conductance changes at different holding potentials were known, and were used to fit a one- (g_K) or a two- (g_K and g_{inc}) parameter model (see Methods). Neurones were divided into two categories based on the presence or absence of a significant g_{inc} during the sEPSC. In seventeen of twenty-four (71%) neurones fibre tract stimulation caused an inward current associated with a 13 ± 3 nS decrease in conductance. The estimated reversal potential of the peak current was -96 ± 3 mV (Fig. 1*A*) and g_{inc} did not make a significant contribution to these responses. The sEPSC in these neurones was considered a conductance decrease type response and has been reported by several other groups (Johnson *et al.* 1980; Grafe *et al.* 1980). In seven of twenty-four (29%) neurones, the sEPSC was associated with no net change in membrane conductance (Fig. 1*B*). The data from these cells were fitted best by a model in which there was an approximately equal contribution of g_K decrease and g_{inc}. The peak sEPSC from these seven neurones did not reverse between −40 and −110 mV and could not be extrapolated to reverse between +50 and −150 mV. These sEPSCs were considered mixed conductance/non-reversing type responses. In four of seven neurones, this relationship did not change during

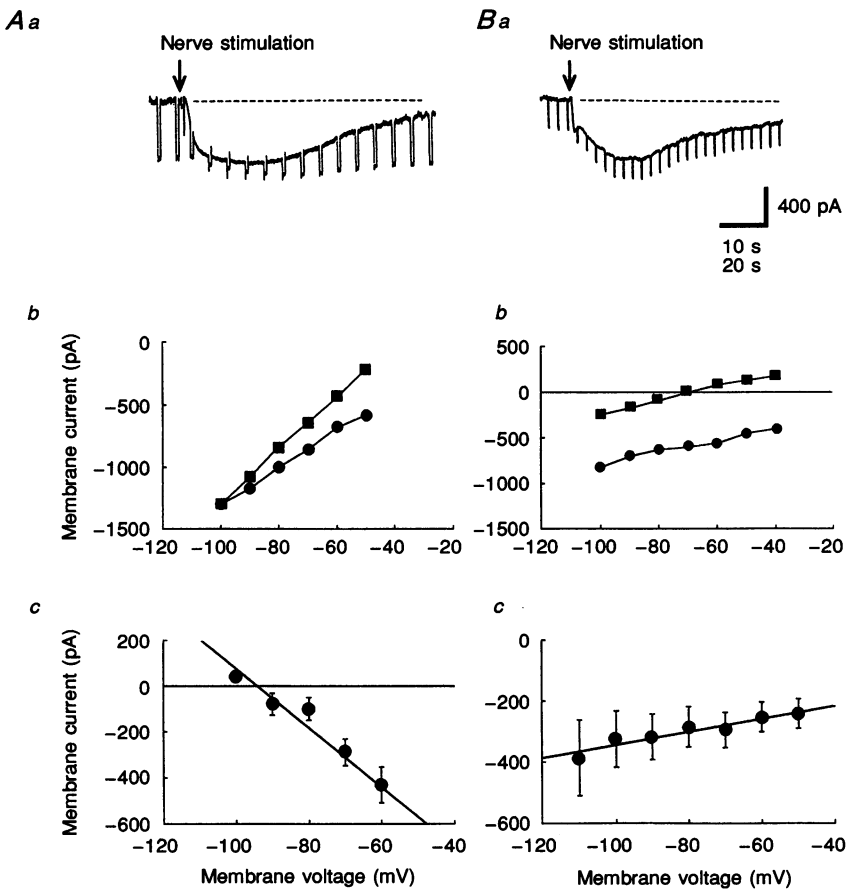

Figure 1. The sEPSC is due to two conductances
Records were obtained 30 min after impalement. Electrical stimulation of interganglionic fibre tracts (arrows) caused a sEPSC. *A a*, sEPSC associated with a conductance decrease ($V_h = -70$ mV, $V_{step} = -90$ mV); *b*, steady-state *I–V* relationship in the same neurone before (■) and at the peak of the sEPSC (●); *c*, average *I–V* for sEPSCs recorded from 7 neurones similar to *A b*; the reversal potential for the sEPSC was -96 ± 3 mV. *Ba*, sEPSC with a biphasic conductance change ($V_h = -70$ mV, $V_{step} = -90$ mV); *b*, steady-state *I–V* relationship in the same neurone before (■) and at the peak of the sEPSC (●); *c*, *I–V* relationship for the sEPSC, recorded from 6 neurones similar to *B b*, did not reverse polarity between −40 and −110 mV.

the time course of the sEPSC. In three of seven neurones, there was a biphasic conductance change, an early conductance decrease was followed by a g_{inc}. The early conductance decrease reversed near −90 mV, which is consistent with this being a decrease in g_K (see Fig. 3*A*).

Senktide mimics the sEPSC

Application of senktide (3 μM) 10–30 min after impalement caused an inward current in 106 of 139 neurones. In 76 of 106 (72%) neurones, senktide-induced currents were associated with a conductance decrease. In 23 of 106 (22%) neurones, the current was associated with either no net conductance change or a conductance decrease followed by a conductance increase. In 12 of 106 (11%) neurones the senktide current was associated with a conductance increase.

An *I–V* relationship was obtained for senktide responses in forty-one AH-neurones at least 30 min after impalement. In these neurones, peak currents and conductance changes at different holding potentials were known, and were used to fit a one- (g_K) or a two- (g_K and g_{inc}) parameter model. In twenty of forty-one (49%) neurones, senktide caused an inward current associated with a 8·4 ± 5 nS decrease in conductance with a reversal potential of −94 ± 2 mV (Fig. 2*A*). These responses did not contain a significant g_{inc}. These actions of senktide on myenteric neurones were considered conductance decrease type responses and are similar to results reported by others (Hanani *et al.* 1988).

In twenty-one of forty-one (51%) neurones, the *I–V* relationships could be fitted best by a two-parameter model in which there was a significant contribution of g_{inc}. In these twenty-one neurones, there were two subgroups. In the first subgroup, eleven of twenty-one neurones, senktide caused a current without a net conductance change. Simulation showed that these currents resulted from a similar contribution from g_K decrease and g_{inc}. The peak currents in these eleven neurones failed to reverse polarity between −40 and −110 mV nor could they be

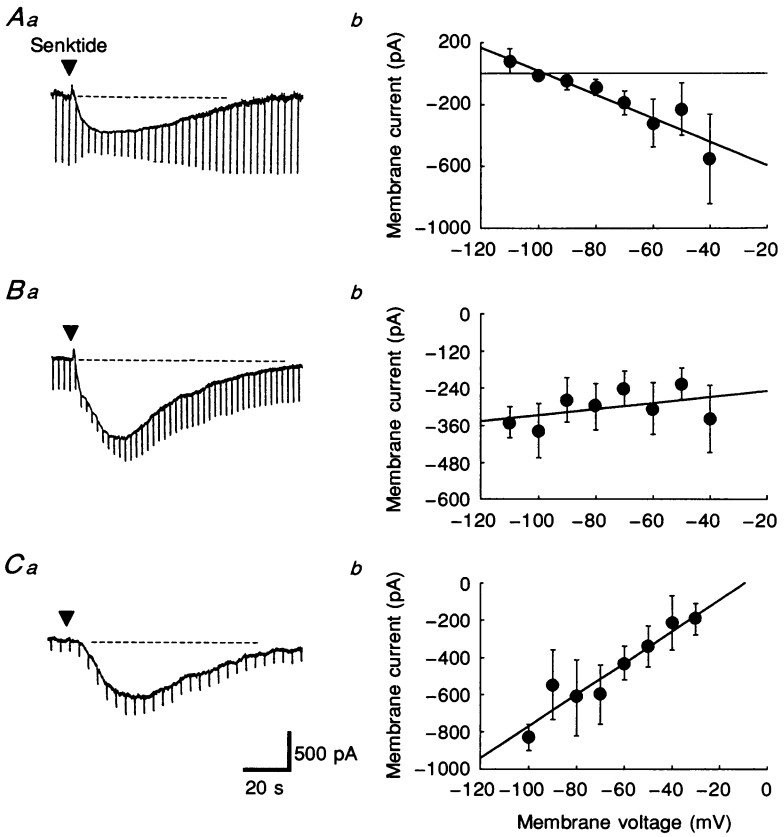

Figure 2. Senktide mimics the sEPSC

Records were obtained 30 min after impalement. Senktide (3 μM) was pressure applied (▼) and caused an inward current; TTX (0·3 μM) present. *A a*, senktide current with a conductance decrease (V_h = −75 mV, V_{step} = −85 mV); *b*, the *I–V* relationship from 10 neurones with similar responses; the reversal potential was −94 ± 2 mV. *Ba*, a senktide current with a biphasic conductance change (V_h = −75 mV, V_{step} = −85 mV); *b*, the *I–V* from 7 neurones with biphasic or unclear conductance changes; responses did not reverse. *Ca*, a senktide current with a g_{inc} (V_h = −65 mV, V_{step} = −85 mV); *b*, *I–V* from 5 neurones with similar responses; the estimated reversal potential was −15 mV.

extrapolated to reverse between +50 and −150 mV. These actions of senktide were considered mixed conductance/non-reversing type responses (Fig. 2*B*). In six of eleven neurones, the relative contribution did not change during the time course of the response. In five of eleven neurones an early g_K decrease was followed by a g_{inc} (see Fig. 5*A*).

In the second subgroup, ten of twenty-one neurones, senktide caused an inward current that was associated with a large g_{inc}-to-g_K ratio. This relationship did not change during the course of the response. The estimated reversal potential of the peak current was −17 ± 3 mV. These actions of senktide were considered conductance increase type responses (Fig. 2*C*).

Forskolin inhibits g_K and reveals a conductance increase during non-reversing slow EPSCs

Forskolin mimicked conductance decrease type sEPSCs. Forskolin (0·01–3 μM) applied by fast flow or superfusion caused a sustained inward current in S- and AH-neurones. In S-neurones, the maximum forskolin (1 μM) current was 132 ± 20 pA (V_h = −70 mV, n = 6) and the reversal potential was −98 ± 5 mV; the forskolin EC_{50} was 0·2 μM. In AH-neurones, the maximum forskolin (1 μM) current was 283 ± 49 pA (V_h = −70 mV, n = 7) and the reversal potential was −111 ± 4 mV; the forskolin EC_{50} was 0·08 μM. It was concluded that forskolin currents were due to inhibition of g_K. These data are similar to those of others (Nemeth *et al.* 1986). Forskolin was applied to neurones with mixed conductance/non-reversing type sEPSCs (Fig. 3*A*). Forskolin (1 μM) caused an inward current in these neurones that was due to a decrease in resting g_K. In the presence of forskolin, these sEPSCs were converted to a conductance increase type response (Fig. 3*B*). The *I–V* relationship for the residual sEPSC yielded an estimated reversal potential of −18·8 ± 8 mV with an increase in chord conductance of 5·9 ± 2 nS (n = 5; Fig. 3*C*).

Before treatment with forskolin, a decrease in g_K accounted for about 70% of the peak total conductance change during the sEPSC and I_K accounted for 30% of the synaptic current (Fig. 4*A*). The small contribution of I_K to the synaptic current can be explained by the small driving force for potassium at a holding potential of −80 mV. During superfusion with forskolin, the contribution of g_K to the sEPSC fell to less than 30% of the absolute conductance change and the peak current was reduced from −200 to −50 pA (Fig. 4*B*). Forskolin did not change g_{inc}. Under these conditions, the current generated by g_{inc}

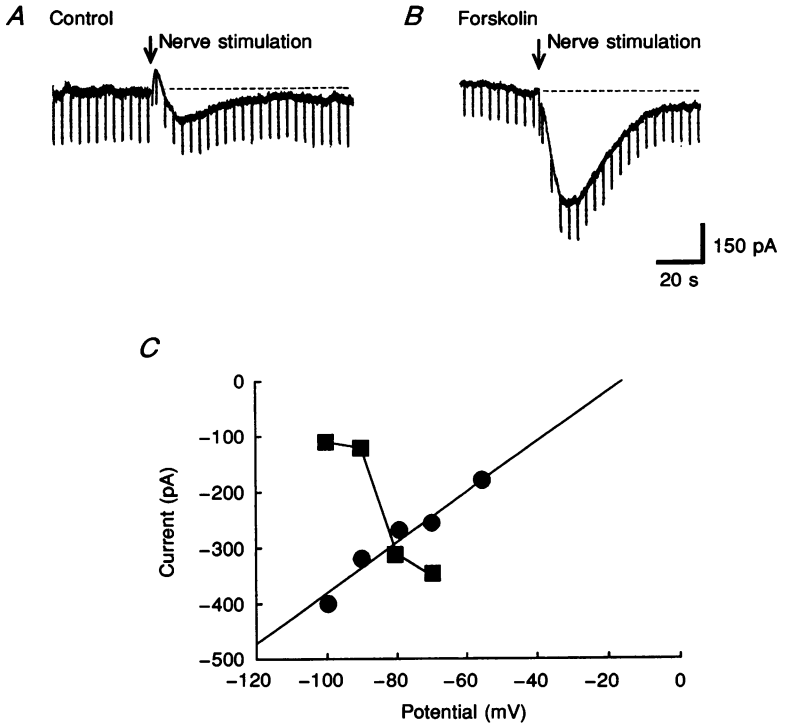

Figure 3. Forskolin occluded g_K but not the conductance increase
A biphasic sEPSC was evoked (arrow) before (*A*) and during (*B*) superfusion with forskolin (1 μM). In control, the sEPSC was composed of an outward current with a conductance decrease followed by an inward current with no net conductance change. In the presence of forskolin (1 μM) the sEPSC was associated with a g_{inc} and the outward current associated with g_K decrease was occluded (V_h = −100 mV; V_{step} = −110 mV). *C*, *I–V* relationship for the sEPSCs in *A* and *B*; control (■) and with forskolin (●). The peak control sEPSC did not reverse (range −35 to −100 mV); in the presence of forskolin the peak current reversed at −16 mV and was associated with a 4·5 nS g_{inc}.

accounted for 90% of the synaptic current (see below for a similar analysis of conductance decrease type responses).

Forskolin inhibits g_K and reveals a conductance increase during non-reversing senktide responses

Forskolin (1 μM) was applied to neurones exhibiting mixed conductance/non-reversing type responses to senktide (Fig. 5*A*) and occluded the g_K decrease caused by senktide (Fig. 5*B*). In the presence of forskolin, these senktide responses were converted to conductance increase type responses. Control senktide responses were -391 ± 78 pA and in the presence of forskolin, the senktide current was -384 ± 81 pA ($V_h = -70$ mV, $P > 0{\cdot}05$, $n = 6$). Currents were estimated to reverse at -14 ± 5 mV ($n = 3$).

The conductance increase (g_{inc}) is a chloride conductance (g_{Cl})

These experiments were carried out under conditions that minimized changes in g_K. Neurones were recorded from in the presence of forskolin (1 μM) and/or caesium (2 mM). In addition, responses were recorded 30 min or more after impalement and V_h was near E_K. All responses studied exhibited a net conductance increase, except where noted.

Ion substitution experiments

Lowering extracellular sodium concentration to 26 mM, by substituting choline chloride (117 mM) for sodium chloride, did not significantly change the senktide response. The control response was -367 ± 83 pA while in the presence of choline chloride the senktide response was -420 ± 117 pA ($V_h = -85$ mV, $P > 0{\cdot}05$, $n = 6$). Reducing extracellular chloride concentration to 13 mM, by substituting sodium isethionate (117 mM) for sodium chloride, reduced senktide-induced currents by 79%. Control responses were -619 ± 99 pA while in the presence of sodium isethionate the current amplitude was -130 ± 51 ($V_h = -80$ mV, $P < 0{\cdot}05$, $n = 4$). Low-chloride solutions also reduced sEPSCs that contained a g_{inc} by 78%. Control sEPSCs were -374 ± 35 pA and in the presence of sodium isethionate, sEPSC amplitude was -83 ± 49 pA ($V_h = -80$ mV, $P < 0{\cdot}05$, $n = 3$). The specificity of the actions of reduced chloride solutions were tested under control conditions (i.e. no forskolin or caesium), using conductance decrease type sEPSCs. The decrease in g_K caused by these sEPSCs was not affected by low-chloride solutions; the control response was 243 ± 78 pA, and in the presence of sodium isethionate,

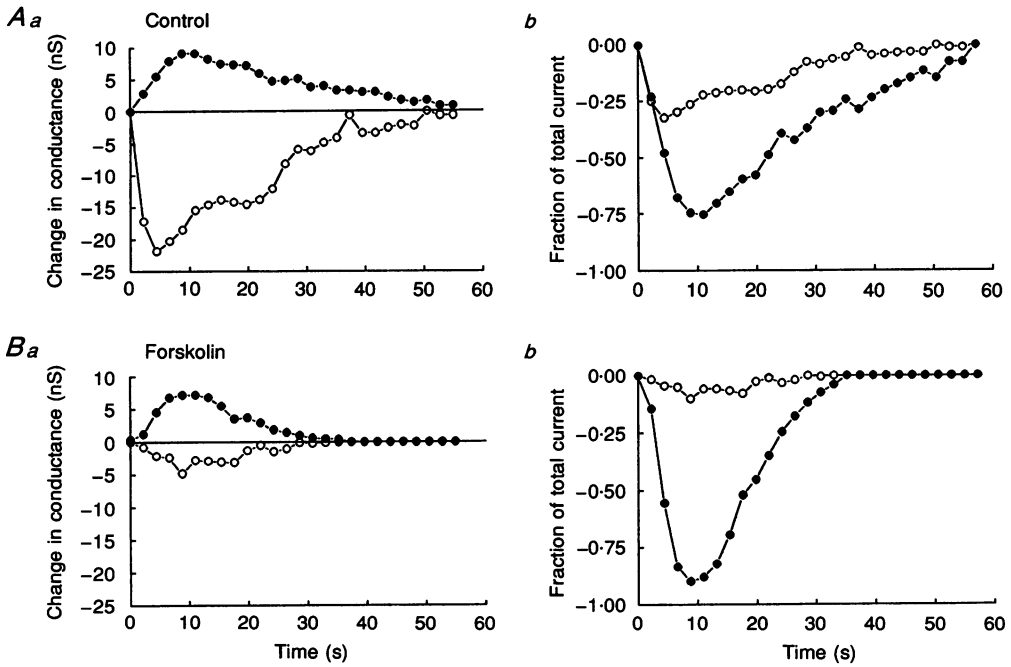

Figure 4. Analysis of the change in conductance associated with a non-reversing sEPSC
Chord conductance and current measurements were taken every 2·2 s during a biphasic sEPSC ($V_h = -80$ mV). The changes in conductance due to g_K and g_{inc} summate and always equal the observed change in conductance. The total combined peak current for each sEPSC was normalized to a value of -1 and the current components were expressed as a fraction of that value. *A a*, decrease in g_K (○) is 5–6 times larger than the g_{inc} (●). *A b*, the same sEPSC was divided into two current components based on the ratio of g_K to g_{inc}. I_K (○) is smaller than the current generated by the g_{inc} (●) due to the small driving force for potassium at -80 mV ($\Delta I = -680$ pA). *Ba*, forskolin (1 μM) specifically occludes the decrease in g_K. *Bb*, the current generated by g_{inc} is effectively isolated ($\Delta I = -500$ pA); symbols as in *A*.

the current amplitude was 191 ± 65 pA ($V_h = -70$ mV, $P > 0.05$, $n = 3$).

An outward chloride current should be increased by reducing extracellular chloride, yet low-chloride solutions reduced the g_{inc}. To determine the cause of this discrepancy, similar studies were performed on $GABA_A$-mediated currents. In normal solutions the reversal potential of the GABA response was −18 ± 2 mV ($n = 10$) while in low-chloride solutions the reversal potential was shifted to +5 ± 6 mV ($n = 5$). On average, when changing from normal to low-chloride solutions, the $GABA_A$ slope conductance was reduced from 27 ± 5 ($n = 10$) to 7 ± 6 nS ($n = 5$). Based on the measured reversal potential of the $GABA_A$ response, and the known extracellular chloride concentration, the intracellular chloride concentration was calculated. When the $GABA_A$ reversal potential was −18 mV and the external chloride concentration was 129 mM, the internal chloride concentration was calculated to be 65 mM. During superfusion with low-chloride solutions, the $GABA_A$ reversal potential was +5 mV and the external chloride concentration was 13 mM, the internal chloride concentration was calculated to be 16 mM. This depletion of internal chloride by low-chloride solutions could account for the reduction of sEPSC and senktide-mediated currents that are associated with significant g_{inc}.

The effect of altered intracellular chloride concentration on the GABA- and senktide-induced chloride currents was also investigated. When recordings were made with a 2 M potassium acetate or a 2 M potassium gluconate electrode in normal Krebs solution, the $GABA_A$ reversal potential was shifted to −39 ± 2 mV ($n = 6$) and the internal chloride concentration was calculated to be 28 mM. Similar experiments were attempted to analyse senktide-induced g_{inc}. With potassium acetate or potassium gluconate electrodes, the chloride equilibrium potential should be shifted from approximately −17 to −39 mV. However, no sEPSCs, senktide or forskolin responses could be recorded in eight of eleven neurones impaled with 2 M potassium acetate electrodes and all four neurones impaled with 2 M potassium gluconate electrodes. In three of eleven neurones impaled with a potassium acetate electrode, senktide caused an inward current associated with a net conductance decrease.

Channel blocker experiments

Tetraethylammonium (TEA) did not reduce senktide-induced currents; the control current was −505 ± 95 pA

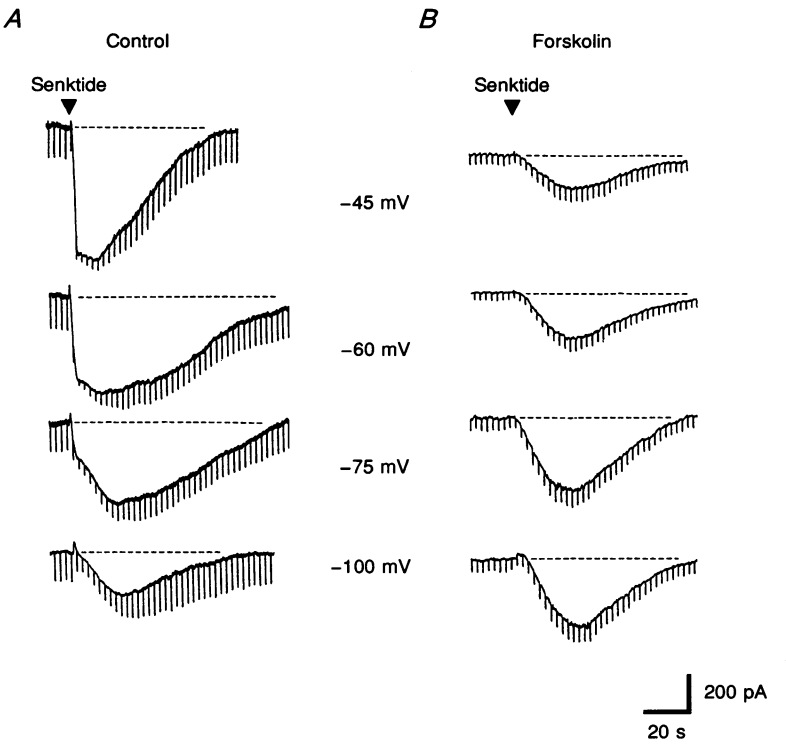

Figure 5. Forskolin occludes g_K and isolates the senktide-induced conductance increase
Senktide (3 μM) was pressure applied (▼) to a neurone and evoked a biphasic response at the indicated holding potentials before (*A*) and during (*B*) superfusion with forskolin (1 μM) ($V_{step} = 20$ mV negative to V_h; 0·3 μM TTX present). *A*, under control conditions the peak current did not reverse. *B*, in the presence of forskolin, the current was monophasic, was estimated to reverse at −10 mV and was associated with a 5·7 nS increase in conductance.

while in the presence of TEA (10 mM) the senktide current was -420 ± 26 pA ($V_h = -70$ mV, $P > 0{\cdot}05$, $n = 4$). Cobalt chloride (2 mM) added to phosphate-free extracellular solution also did not reduce senktide-induced currents. The control senktide response was -529 ± 59 pA while in the presence of cobalt, the senktide current was -485 ± 51 pA ($V_h = -80$ mV, $P > 0{\cdot}05$, $n = 4$). Picrotoxin (30 μM) added to the Krebs solution did not affect senktide-induced currents; in two neurones, the control responses were -460 and -500 pA and in the presence of picrotoxin, the current amplitudes were -410 and -600 pA ($V_h = -80$ mV).

Niflumic acid (NFA) and mefenamic acid (MFA) inhibit GABA and senktide-induced currents

NFA and MFA are reversible blockers of some chloride conductances (White & Aylwin, 1990). NFA (10–300 μM) and MFA (10–300 μM) inhibited $GABA_A$-mediated responses. The $GABA_A$ response was inhibited by $77 \pm 5\%$ following superfusion with 300 μM NFA ($V_h = -70$ mV, $n = 3$, $P < 0{\cdot}05$) and by $84 \pm 4\%$ following superfusion with 300 μM MFA ($V_h = -70$ mV, $n = 4$, $P < 0{\cdot}05$). The NFA EC_{50} was 72 μM ($n \geqslant 3$) and the MFA EC_{50} was 21 μM ($n \geqslant 3$).

NFA (100 μM) caused a $3{\cdot}5 \pm 1{\cdot}3$ nS increase in g_K ($n = 4$) and NFA (300 μM) caused a $13{\cdot}4 \pm 2{\cdot}7$ nS increase in g_K ($n = 4$). Similar data were obtained with MFA. In order to minimize activation of g_K, concentrations of 100 μM MFA and NFA was chosen to study the effects of chloride conductance blockade on the senktide-induced g_{inc}.

Senktide (3 μM) was pressure applied and only responses which exhibited a net conductance increase were used. NFA (100 μM) and MFA (100 μM), both applied by superfusion, caused a significant block of the senktide-induced current (Fig. 6). NFA caused a decrease in peak current from -471 ± 106 to -123 ± 27 pA ($V_h = -85$ mV, $P < 0{\cdot}05$, $n = 4$). MFA caused a decrease in peak current from -651 ± 103 to -246 ± 57 pA ($V_h = -85$ mV, $P < 0{\cdot}05$, $n = 4$). The specificity of the fenamates was tested under control conditions (i.e. no forskolin or caesium). Neither the sEPSC nor the senktide-induced decrease in g_K were affected by the addition of MFA (300 μM). MFA also did not inhibit fEPSPs recorded from S-neurones. Control fEPSPs were $19{\cdot}8 \pm 0{\cdot}9$ mV, and in the presence of 100 μM

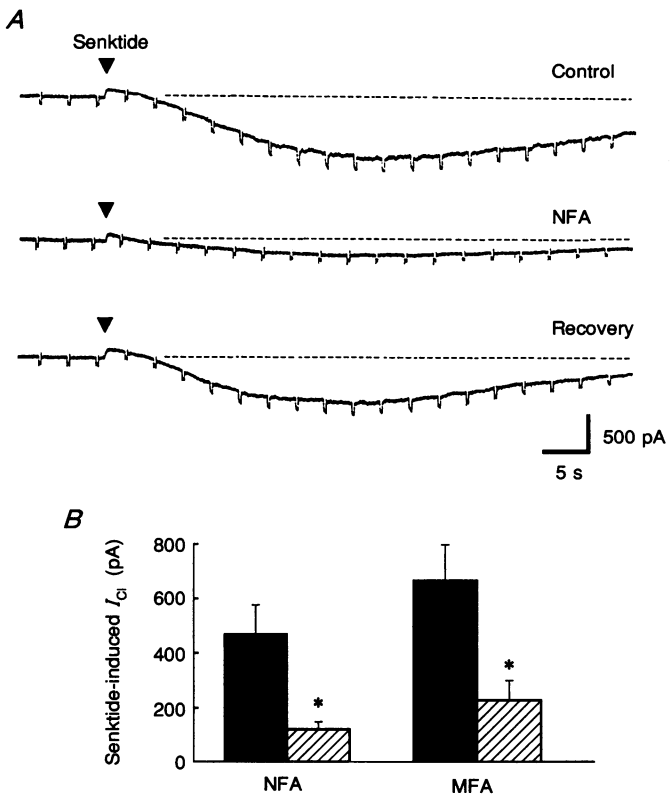

Figure 6. Niflumic acid (NFA) and mefenamic acid (MFA) inhibit senktide-induced currents
A, senktide (3 μM) was pressure applied (▼) and caused an inward current ($V_h = -85$ mV, $V_{step} = -105$ mV). Forskolin (1 μM) was present to occlude senktide-induced inhibition of g_K (0·3 μM TTX was present). NFA (100 μM) was added to the superfusing Krebs solution 2 min prior to the second senktide response. *B*, NFA and MFA inhibit senktide-induced currents associated with a g_{inc}. Asterisks indicate significant depression of senktide-induced currents in the presence of NFA ($n = 4$) and MFA ($n = 4$) ($P < 0{\cdot}05$). ■, control; ▨, fenamate.

MFA fEPSPs were $20{\cdot}5 \pm 0{\cdot}8$ mV ($V_h = -90$ mV, $P > 0{\cdot}05$, $n = 3$).

NFA and MFA are potent non-steroidal anti-inflammatory drugs. The possibility that inhibition of cyclo-oxygenase was responsible for inhibition of chloride currents was tested. Indomethacin (30–300 μM) did not inhibit the senktide-induced g_{inc}; the control response was -160 ± 72 pA and in the presence of indomethacin, the senktide current was -193 ± 64 pA ($V_h = -80$ mV, $P > 0{\cdot}05$, $n = 2$). Indomethacin (100–300 μM) also did not alter the $GABA_A$ response, the control response was -419 ± 95 pA and in the presence of indomethacin, the GABA current was -384 ± 70 pA ($V_h = -70$, $P > 0{\cdot}05$, $n = 3$). Based upon these and the above observations, we conclude that the senktide-induced g_{inc} and most likely the sEPSC-induced g_{inc} are a chloride conductance (g_{Cl}).

A g_{Cl} increase is present in sEPSCs and senktide responses which exhibit an apparent conductance decrease

Distribution of g_K and g_{Cl} in sEPSCs

Forskolin was used to determine the relative distribution of g_K and g_{Cl} in sEPSCs exhibiting a net conductance decrease. Changes in chord conductance between the holding potential (V_h −55 to −70 mV) and the step command ($V_{step} = 20$ mV negative to V_h, every 2·2 s) were analysed. Under these conditions, g_{Cl} would become more prominent as was the case with non-reversing sEPSCs (see Fig. 3). In twenty of thirty-five (57%) neurones, forskolin occluded sEPSCs by 83 ± 7%. This is consistent with these sEPSCs being mediated by a decrease in g_K only. In fifteen of thirty-five (43%) neurones, forskolin potentiated sEPSCs by 42 ± 25% and the conductance change during the sEPSC was converted to either an unclear conductance change or an increase in chloride conductance.

Relative proportions of g_K to g_{Cl} during the sEPSC

In order to determine the relative proportions of g_K and g_{Cl} contributing to sEPSCs exhibiting a net conductance decrease, changes in chord conductance between the holding potential (V_h −55 to −70 mV) and the step command ($V_{step} = 20$ mV negative to V_h, every 2·2 s) were analysed. At the peak of the sEPSC, the absolute conductance change was composed of a 91 ± 3% decrease in g_K and a 9 ± 3% increase in g_{Cl}. The times to peak for I_K and I_{Cl} were 8 ± 1 and 20 ± 2 s, respectively. The proportion of g_K to g_{Cl} at the time of peak I_K ($g_K = 92 \pm 2$% , $g_{Cl} = 8 \pm 2$%) was different from at the time of peak I_{Cl} ($g_K = 81 \pm 3$% , $g_{Cl} = 19 \pm 3$%; Fig. 7*A*).

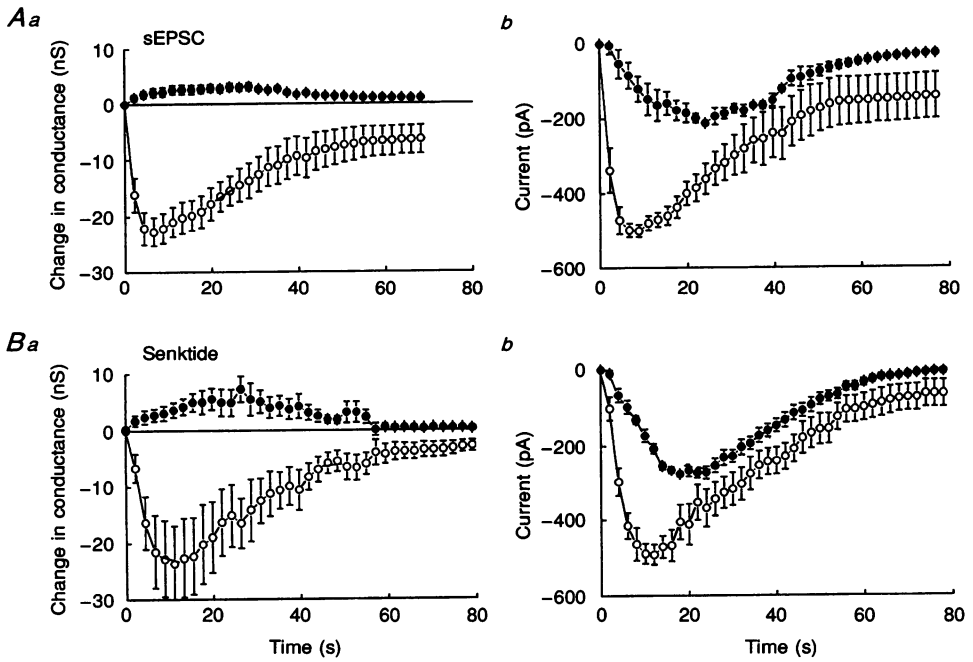

Figure 7. K^+ and Cl^- components isolated from sEPSCs and senktide responses that were associated with an apparent conductance decrease

V_h ranged from −55 to −75 mV (0·3 μM TTX present in *B*). Current and chord conductance measurements were made every 2·2 s. *A a*, average conductance changes associated with the sEPSC. g_K (○) was 90% of the Δg while g_{Cl} (●) was 10% ($n = 8$); *b*, currents were averaged and the value of each component plotted; ○, I_K; ●, I_{Cl}. *Ba*, average conductance changes associated with the senktide response were similar to the sEPSC ($n = 10$); *b*, average senktide-induced currents; symbols as in *A*.

Relative proportions of g_K to g_{Cl} during the senktide response

Conductance changes associated with conductance decrease type senktide responses were analysed in the presence of TTX (0·3 μM). Senktide currents and conductances were measured as described above (V_h −60 to −70 mV, $n = 10$). At the peak of the senktide-induced current, the absolute conductance change was composed of a 79 ± 12% decrease in g_K and a 21 ± 12% increase in g_{Cl}. The times to peak for I_K and I_{Cl} were 12 ± 2 s and 20 ± 1 s, respectively. The proportion of g_K to g_{Cl} at the time of peak I_K ($g_K = 81 \pm 11\%$, $g_{Cl} = 19 \pm 11\%$) was different from at the time of peak I_{Cl} ($g_K = 66 \pm 10\%$, $g_{Cl} = 34 \pm 10\%$). The rate of rise for I_K (rate constant, $\tau = 7{\cdot}2$ s) was faster than the rate of rise for I_{Cl} ($\tau = 16{\cdot}6$ s) while the rate of decay for I_K ($\tau = 27{\cdot}3$ s) was slower than that of I_{Cl} ($\tau = 18{\cdot}6$ s; Fig. 7*B*).

DISCUSSION

The sEPSC is a multiconductance event

It is known that sEPSPs in enteric neurones frequently do not reverse polarity at E_K and there is often no net resistance change during the response. While these data have been attributed to actions of neurotransmitters or drugs at electrically distant sites on neurones, Shen & Surprenant (1993) have recently shown that in submucosal neurones of guinea-pig ileum, agonist-induced currents and sEPSCs are due to simultaneous inhibition of g_K and activation of a non-specific cation conductance. This conclusion was based on the insensitivity of currents to anthracene-9-carboxylic acid and chloride substitution, and current inhibition by extracellular sodium substitution. Simultaneous activation of a cation conductance and inhibition of g_K would account for the inability to reverse the sEPSC or agonist-induced current in these neurones. However, in the present study it has been shown that in a subset of myenteric neurones of guinea-pig ileum, the sEPSC is due to inhibition of g_K and activation of a chloride conductance (g_{Cl}).

The conclusion that a g_{Cl} is involved in the sEPSC of myenteric neurones is based on the findings that some sEPSCs did not reverse at E_K and did not display a net conductance decrease. Additionally, some sEPSCs clearly contained two phases of conductance change with an early decrease in g_K followed by a slower developing increase in g_{Cl}.

Senktide was used to mimic the sEPSC. Most senktide-induced currents were associated with a g_K decrease, but, as with the sEPSC, in some neurones senktide-induced currents could not be attributed to a g_K decrease alone. Senktide responses often did not reverse polarity at E_K and senktide-induced currents were frequently biphasic with an early g_K decrease followed by a later increase in g_{Cl}. In addition, some senktide-induced currents were associated with only an increase in g_{Cl} and these currents reversed at −17 mV.

The conductance increase is g_{Cl}

Forskolin mimics the sEPSC-induced decrease in g_K in myenteric neurones (Palmer *et al.* 1987). Forskolin was used to occlude the decrease in g_K caused by mediators of the sEPSC and senktide, and thus any remaining currents induced by these stimuli would be due to another ionic mechanism. Caesium chloride was also used to block the hyperpolarization-activated cation current (I_H) which is present in some myenteric neurones (Galligan *et al.* 1990). In addition, recordings were obtained more than 30 min after impalement during which time there was the gradual decrease in membrane conductance that commonly occurs in myenteric neurones (Surprenant *et al.* 1987; Wood, 1989). Under these conditions any senktide- or sEPSC-induced changes in g_K would be minimized.

A reversal potential for a transmitter- or agonist-induced current between −25 and −10 mV in myenteric neurones is consistent with an increase in a non-specific cation conductance or a chloride conductance (Cherubini & North, 1984; Wood, 1989; Galligan *et al.* 1989, 1990). It is unlikely that the conductance increase observed here is due to a cation conductance as substitution of external sodium by choline, or addition of caesium chloride or cobalt chloride to the extracellular solution, did not affect the senktide-induced currents.

Lowering extracellular chloride reduced currents associated with g_{Cl}. In peripheral neurones at rest, the intracellular concentration of chloride is such that a lowered extracellular chloride concentration should augment the efflux of chloride from neurones. Akasu, Nishimura & Tokimasa (1990) demonstrated this effect in rabbit pelvic parasympathetic ganglia where there is a calcium-activated chloride conductance. We studied $GABA_A$-mediated chloride currents in myenteric AH-neurones in order to clarify the effects of reduced extracellular chloride solutions on chloride conductances. Prolonged (> 5 min) treatment of preparations with reduced chloride solutions decreased GABA-induced currents and caused a positive shift in the GABA reversal potential. This effect on chloride efflux has been described in sympathetic neurones where it was found that reduced extracellular chloride depleted intracellular chloride over time, or after repeated applications of GABA (Adams & Brown, 1975). The intracellular chloride concentration in AH-neurones was calculated based on the reversal potential of GABA-induced currents and the known extracellular chloride concentration. Intracellular chloride concentration is reduced from 65 mM at rest (recorded with a 2 M KCl electrode) to approximately 16 mM during

superfusion with reduced chloride solutions. The decrease in driving force for chloride would cause an approximately 50% reduction in chloride-mediated currents at a holding potential of −80 mV. These data can explain the reduction of senktide-induced currents and sEPSCs by reduced chloride solutions at similar holding potentials.

Senktide and $GABA_A$ currents were blocked by the fenamates, niflumic and mefenamic acid. These drugs block anion transport (Cousin & Motais, 1979) and anion channels (White & Aylwin, 1990). Fenamates can also block cation conductances (Gogelein, Dahlem, Englert & Lang, 1990), but not cation transport (Cousin & Motais, 1979). It is unlikely that the fenamates were blocking cation channels in the present study as fEPSPs in S-neurones were unaffected by these drugs. Indomethacin, a potent non-steroidal anti-inflammatory drug, was used in concentrations exceeding those needed to cause maximal inhibition of cyclo-oxygenase *in vitro* (Stutts, Henke & Boucher, 1990). Indomethacin did not affect either senktide- or GABA-induced currents, and thus it is unlikely that cyclo-oxygenase inhibition is responsible for the fenamate effects on myenteric neurones reported here.

At high concentrations (100–300 μM) the fenamates caused an increase in g_K. Toro, Ottolia, Olcese & Stefani (1993) have shown that low concentrations of niflumic acid and flufenamic acid cause an increase in the open probability of calcium-activated potassium channels situated in a lipid bilayer. Also, Farrugia, Rae & Szurszewski (1993) have found that flufenamic acid and mefenamic acid caused a dose-dependent opening of a delayed rectifier-like potassium channel in isolated smooth muscle cells from canine jejunum.

Characterization of g_{Cl}

Slow EPSCs, recorded more than 30 min after impalement and in which an I–V relationship had been measured, were categorized based upon a significant contribution of g_{Cl}. These results indicated that up to 29% of these sEPSCs contained an increase in g_{Cl}. In most neurones, an I–V relationship was not measured, but similar analysis could be used to suggest a contribution of g_{Cl}. Responses in which there was a net conductance decrease were examined using forskolin to occlude changes in g_K, and unmask g_{Cl}. These data indicate that 43% of sEPSCs initially associated with a net conductance decrease may also contain a significant increase in g_{Cl}. When measured without forskolin these sEPSCs contained approximately a 9:1 ratio of g_K to g_{Cl}.

Senktide currents, in the presence of TTX, were used to study I_{Cl} and I_K in detail. On average, the time constant for the rate of rise of I_K (7 s) was faster than that for I_{Cl} (17 s). Thus, when the contributions of the two conductances are equal, a clear biphasic conductance change is observed. When the difference in time course of these conductances is less prominent (due to a slower rate of rise for I_K), the observed current is associated with no net conductance change. The time constant of decay for I_K was slower than for I_{Cl}. The apparently slower rate may be due in part to the irreversible activation of I_K (i.e. decrease in g_K) that is common during impalement of myenteric neurones (Surprenant *et al.* 1987).

The time to peak for senktide- and sEPSC-induced I_{Cl} were both slower than the time to peak of I_K. The time to peak of the total evoked current in both cases, was more closely related to changes in g_K. These data indicate that changes in g_{Cl} are involved in the maintenance of the sEPSC, while the peak current is due mainly to changes in g_K. The measurement of peak current, which is commonly used to construct I–V relationships, may significantly under-represent the contribution of a change in g_{Cl}.

Forskolin was used to mimic the decrease in g_K seen during senktide-induced currents and sEPSCs. The effectiveness of forskolin in isolating g_{Cl} also indicates that forskolin-sensitive pathways are not directly responsible for activation of g_{Cl}. Myenteric neurones respond to stimulation of signal transduction pathways other than those that are cAMP–protein kinase A-dependent. For example, myenteric neurones depolarize upon activation of diacylglycerol–PKC-dependent pathways (North *et al.* 1987; Bertrand & Galligan, 1993*a*). It is likely, then, that the direct activation of g_{Cl} is by intracellular transduction mechanisms other than cAMP–protein kinase A.

Physiological significance of an increase in g_{Cl}

The resting input resistance of myenteric neurones *in vivo* is expected to be higher than that recorded *in vitro* using intracellular electrodes. The quality of electrode impalement directly affects the measured input resistance of a neurone. Original estimates of myenteric neurone input resistances were between 20 and 50 MΩ (Nishi & North, 1973). Estimates have varied considerably between studies, but on average have been moved to higher values as intracellular recording techniques have improved (see Wood, 1989, for review). Whole-cell patch clamp recordings from cultured myenteric neurones have yielded measured input resistances for AH- and S-neurones of 234 ± 12 and 345 ± 23 MΩ, respectively (authors' unpublished data). These data are similar to others (Tatsumi, Costa, Schimerlik & North, 1990; Baiden, Zholos, Shuba & Wood, 1992). This implies that *in vivo*, many potassium channels which set the membrane potential may be closed and unavailable to mediate sEPSPs. Under these conditions an increase in g_{Cl} could account for a larger proportion of the inward current during the sEPSP. A decrease in g_K and an increase in g_{Cl} is an ideal combination for exciting neurones. During such a response, sodium and calcium gradients remain intact, thus preserving the neurones' ability to generate fEPSPs and action potentials.

Conversely, large increases in cation conductance could deplete the transmembrane sodium gradient leading to inhibition of synaptic transmission.

In conclusion, these data indicate a role for an increase in g_{Cl} in slow synaptic transmission within the myenteric plexus. This g_{Cl} can be activated by agonists acting at neuronal tachykinin receptors, but is not directly activated by the cAMP–PKA second messenger system.

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