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A SLOW CALCIUM-DEPENDENT CHLORIDE CURRENT IN RHYTHMIC HYPERPOLARIZATION IN NEURONES OF THE RABBIT VESICAL PELVIC GANGLIA

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SUMMARY

1. Voltage-clamp recordings were made from neurones of vesical pelvic ganglia isolated from the rabbit urinary bladder. A rhythmic outward current, I_{SH}, which corresponds to the spontaneous hyperpolarization, occurred at fairly constant intervals in fifty-eight of eighty-four neurones superfused with Krebs solution. The peak amplitude of the I_{SH} was $0{\cdot}5 \pm 0{\cdot}2$ nA ($n = 48$; mean ± S.E.M.).

2. The I_{SH} was eliminated in a Krebs solution containing nominally zero calcium and 12 mM-magnesium. Lowering the temperature of the superfusing solution from 36 to 22 °C also inhibited the occurrence of the I_{SH}.

3. Bath application of caffeine increased the frequency of I_{SH}. In contrast, ryanodine and procaine reversibly blocked I_{SH}.

4. In thirty-four of fifty-eight neurones, the I_{SH} was composed of two current components, an initial fast I_{SH} with duration of 1–10 s and a slow I_{SH} lasting 15–60 s. In the remaining twenty-four neurones, I_{SH} showed only the fast component.

5. The fast I_{SH} was associated with an increased membrane conductance and the slow I_{SH} was associated with a decreased membrane conductance. The reversal potentials of the fast and the slow I_{SH} were -88 ± 7 mV ($n = 4$) and -30 ± 6 mV ($n = 4$), respectively.

6. Tetraethylammonium (5 mM) and barium (1 mM) blocked the fast I_{SH} but not the slow I_{SH}. Intracellular caesium injected by ionophoresis through a Cs^+-filled microelectrode blocked the fast I_{SH}, without affecting the slow I_{SH}. Apamin and (+)-tubocurarine selectively suppressed the fast component of the I_{SH}.

7. Substitution of isethionate (67 mM) for chloride increased the amplitude of the slow I_{SH} and shifted the reversal potential of the slow I_{SH} to $+1 \pm 8$ mV ($n = 5$). A slow I_{SH} with amplitude of 0·1–1 nA and was still observed in a low-sodium (26·2 mM) solution. The stilbene derivative, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS), a chloride channel blocker, suppressed the slow I_{SH}.

8. These results suggest that I_{SH} is composed of two distinct calcium-dependent currents, a fast I_{SH} produced by activation of potassium conductance and a slow I_{SH} produced by inactivation of chloride conductance.

9. The after-hyperpolarization (AHP) following the action potential was also composed of apamin-sensitive and insensitive spontaneous hyperpolarizing oscil-

lations. The apamin-insensitive component of I_{AHP} was increased by lowering external chloride activity, while it was depressed by SITS.

INTRODUCTION

Intracellular calcium is known to regulate a potassium conductance which is referred to as the calcium-dependent potassium conductance ($g_{K,Ca}$) in neuronal tissues (Meech, 1972, 1978). At least two types of calcium- and voltage-dependent potassium current ($I_{K,Ca}$) exist in autonomic neurones, a fast current which contributes in part to repolarization of the action potential and to the fast after-hyperpolarization (Adams, Constanti, Brown & Clark, 1982; MacDermott & Weight, 1982; Tanaka, Minota, Kuba, Koyano & Abe, 1986; Lancaster & Pennefather, 1987) and a slow after-current producing a slow after-hyperpolarization (Suzuki & Kusano, 1978; McAfee & Yarowsky, 1979; Morita, Koketsu & Kuba, 1980; Morita, North & Tokimasa, 1982; Kuba, Morita & Nohmi, 1983; Hirst, Johnson & van Helden, 1985; Pennefather, Lancaster, Adams & Nicoll, 1985; Cassell & McLachlan, 1987). A calcium-dependent potassium current also contributes to resting membrane potential in neurones of myenteric plexus (Grafe, Mayer & Wood, 1980) and vesical pelvic ganglia (Nishimura, Tokimasa & Akasu, 1988).

Caffeine causes a rhythmic hyperpolarization in autonomic neurones (Kuba & Nishi, 1976; Skok, Storch & Nishi, 1978; Suzuki & Kusano, 1978, 1983; Kuba, 1980), due to a periodic activation of a potassium conductance triggered by a rise in the concentration of intracellular calcium ions (Smith, MacDermott & Weight, 1983; Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988). Spontaneous hyperpolarizing oscillation (SH) of the resting membrane potential has been found to occur at fairly constant intervals in neurones of rabbit vesical pelvic ganglia in physiological solutions (Nishimura *et al.* 1988). The action potential also triggers the occurrence of the SH. Current-clamp study has shown that a calcium-dependent conductance, probably the $g_{K,Ca}$, contributes to the activation of the SH (Nishimura *et al.* 1988).

The purpose of the present study is to determine the ionic mechanism of the SH using the voltage-clamp technique. The results indicate that the spontaneous outward current (I_{SH}), which corresponded to the SH, is composed of two current components, a fast I_{SH} produced by activation of the calcium-dependent potassium conductance and a slow I_{SH} produced by inhibition of the calcium-dependent chloride conductance. A preliminary report of this work has been published (Nishimura, Tokimasa & Akasu, 1989).

METHODS

Male white rabbits weighing 2·0–3·0 kg were anaesthetized with sodium pentobarbitone (40–50 mg/kg I.V.). Isolation of vesical pelvic ganglia has been described previously (Nishimura *et al.* 1988). After removal of the ganglia, rabbits were killed by intravenous injection of an excess dose of pentobarbitone. Connective tissue surrounding the ganglia was removed as much as possible by dissection, so that the cells were easier to penetrate and easier to expose to applied solutions. Ganglia were pinned onto Sylgard at the bottom of a perfusion chamber and continuously superfused with Krebs solution having the following composition (mM): NaCl, 117;

KCl, 4·7; $CaCl_2$, 2·5; $MgCl_2$, 1·2; NaH_2PO_4, 1·2; $NaHCO_3$, 25; and glucose, 11. Calcium-free solution contained nominally zero calcium and 12 mM-magnesium. Low-chloride solution was made by replacement of 67 mM-sodium chloride with equimolar sodium isethionate. In a low-sodium solution, 117 mM-sodium chloride was replaced with equimolar tetraethylammonium chloride or choline chloride. In some experiments, caesium (2 mM) was added to the superfusing solution to block the inward rectifier current (Nishimura *et al.* 1988). Solution was gassed with 5% CO_2–95% O_2 and preheated to 35–37 °C at the recording site until otherwise specified. The temperature of the solution was continuously monitored by a thermistor.

Current- and voltage-clamp experiments were performed with an Axoclamp 2A (Axon Instruments, Burlingame, CA, USA) (Akasu, Nishimura & Tokimasa, 1990). For current-clamp experiments, ganglion neurones were impaled with glass microelectrodes filled with 3 M-potassium chloride or 2 M-caesium chloride. Tip resistances ranged between 30 and 50 MΩ. For discontinuous single-electrode voltage clamp, similar glass microelectrodes were used. Sampling frequency was between 3 and 5 kHz (70–30 duty cycle) and gain was between 3 and 8%. The head stage of the amplifier was monitored on a separate oscilloscope. The signal from the microelectrode was displayed on an oscilloscope with a digitized memory (Nihon Kohden, VC-11) and recorded on a pen-writing chart recorder (Nihon Kohden RJG-3022).

Drugs used were: tetrodotoxin (TTX) purchased from Sankyo; tetraethylammonium chloride (TEA) from Tokyo Kasei; 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid, di-sodium.$3H_2O$ (SITS) from Research Organics, Inc.; (+)-tubocurarine hydrochloride and apamin from Sigma; procaine hydrochloride from Daiichi Seiyaku (Japan); caffeine from Wako Pure Chemicals (Japan); 3-isobutyl-1-methylxanthine from Aldrich; ryanodine from Calbiochem Co.; lidocaine hydrochloride was from Research Biochemicals, Inc.

Data are expressed as means ± S.E. of mean.

RESULTS

Spontaneous outward current (I_{SH})

The resting membrane potential of vesical pelvic neurones examined 10 min after an impalement with a KCl-filled microelectrode ranged from −50 to −76 mV (−57 ± 6 mV, $n = 74$; mean ± S.E.M.). The membrane input resistance was 83 ± 6 MΩ ($n = 74$) at rest. Rhythmic hyperpolarizing oscillations of the resting membrane potential, the spontaneous hyperpolarization (SH), were recorded in Krebs solution (Fig. 1*A*). The SH occurred at fairly constant intervals ranging between 30 s and 5 min in individual neurones (see also Nishimura *et al.* 1988). The SH had an initial rapid phase followed by a slower hyperpolarizing phase (Fig. 1*A*). The input membrane resistance was decreased during the initial rapid phase, while it was increased during the slower phase (Fig. 1).

Spontaneous outward current corresponding to the SH (I_{SH}) was recorded from fifty-eight of eighty-four neurones voltage clamped at −50 to −65 mV (Fig. 1). The remaining twenty-six neurones showed no I_{SH}. The peak amplitude of I_{SH} ranged between 0·1 and 1·0 nA (0·5 ± 0·2 nA, $n = 48$). Although the interval between the appearance of I_{SH} varied within individual neurones, it remained fairly constant at a given holding potential. In thirty-four neurones, I_{SH} was composed of initial rapid and following slower phases. The initial rapid phase of the I_{SH} was associated with increased membrane conductance. In contrast, the membrane conductance was decreased during the slowly decaying current component (Fig. 1). In twenty-four neurones, the I_{SH} was composed of only the rapid current component associated with increased membrane conductance (see Fig. 7).

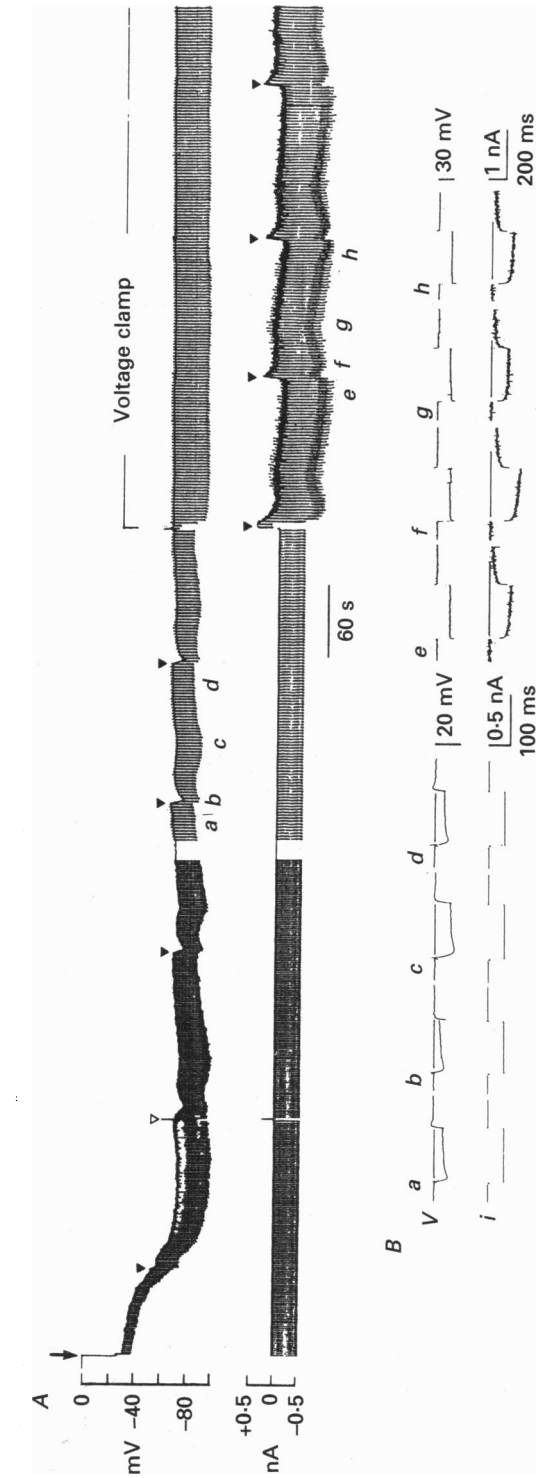

Fig. 1. Spontaneous hyperpolarization (SH) and outward current (I_{SH}) recorded from a single ganglion cell. *A*, the arrow indicates the insertion of a glass microelectrode filled with 3 M-KCl. Filled triangles indicate the occurrence of the SH and I_{SH}. A depolarizing current pulse (lower trace) was applied through the recording microelectrode to produce an action potential (open triangle). Hyperpolarizing electrotonic potentials (upper trace) were produced by anodal currents with amplitude of 0·5 nA (lower trace). Under the voltage-clamp procedure (holding potential −62 mV), hyperpolarizing voltage commands with a duration of 200 ms were applied to the ganglion cell to measure the membrane conductance. *B*, expanded records of the hyperpolarizing electrotonic potential (*a–d*) and the inward current produced by hyperpolarizing voltage jumps (*e–h*). Each record was taken at the time marked by respective letters in *A*.

Calcium-dependent properties of I_{SH}

Removal of extracellular calcium

It has been shown that removal of extracellular calcium depolarized the ganglion neurone and increased membrane resistance (Nishimura *et al.* 1988). The calcium

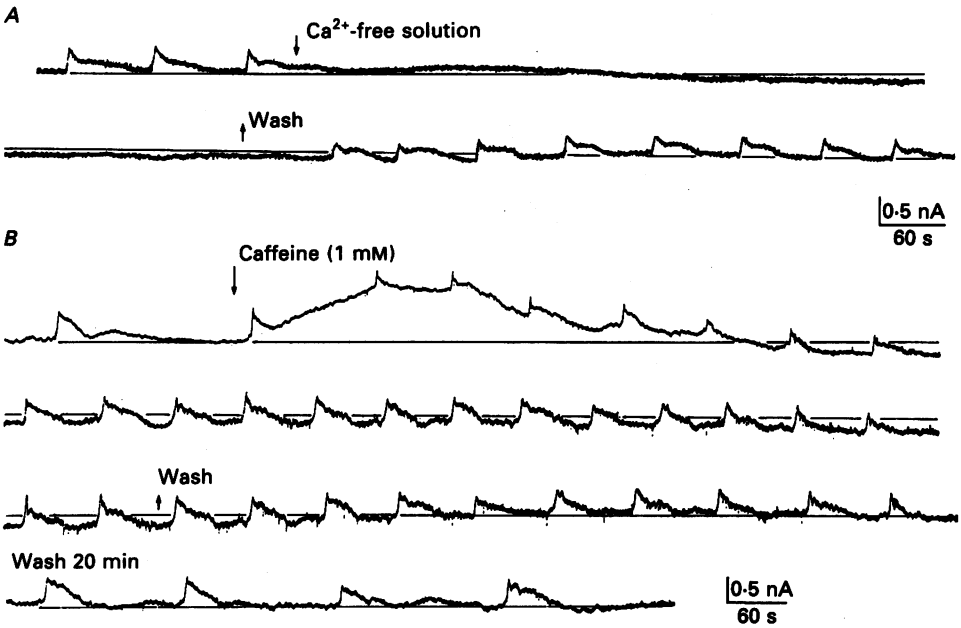

Fig. 2. Effects of removal of extracellular calcium (*A*) and addition of caffeine (1 mM) (*B*) in the I_{SH} recorded from ganglion cells. *A*, the calcium-free Krebs solution contained zero calcium and 12 mM-magnesium. The resting membrane potential was −55 mV. Upper and lower traces represent consecutive recordings of the membrane current. Downward and upward arrows indicate the period of application of the calcium-free solution. *B*, effect of caffeine (1 mM) on the I_{SH}. Holding membrane potential was −62 mV. Upper three traces were consecutive recordings of the I_{SH}. Caffeine was applied to the superfusing solution between the two arrows. Lowermost trace was taken 20 min after removal of caffeine from the bath. Note that caffeine increased the frequency of I_{SH}.

dependence of I_{SH} was, therefore, examined under the voltage-clamp condition. Treatment of a pelvic ganglion cell with a Krebs solution containing nominally zero calcium and 12 mM-magnesium (Ca^{2+}-free solution) caused an inward shift of the holding current and completely blocked the occurrence of I_{SH} within 2 min ($n = 8$; Fig. 2*A*). When the external solution was switched to normal Krebs solution, I_{SH} was restored within 3–5 min.

Caffeine

Caffeine and theophylline produce rhythmic hyperpolarizations of the resting membrane potential in autonomic neurones (Kuba & Nishi, 1976; Skok *et al.* 1978; Suzuki & Kusano, 1978; Kuba, 1980; Smith *et al.* 1983; Nishimura *et al.* 1988) by increasing intracellular calcium ions (Smith *et al.* 1983; Lipscombe *et al.* 1988). The

application of caffeine (1–3 mM) to pelvic ganglion neurones produced a transient outward current followed by a small inward current which lasted throughout its application. I_{SH} increased in frequency in the presence of caffeine (1 mM; $n = 5$). Caffeine produced no significant change in the peak amplitude of I_{SH} (Fig. 2*B*).

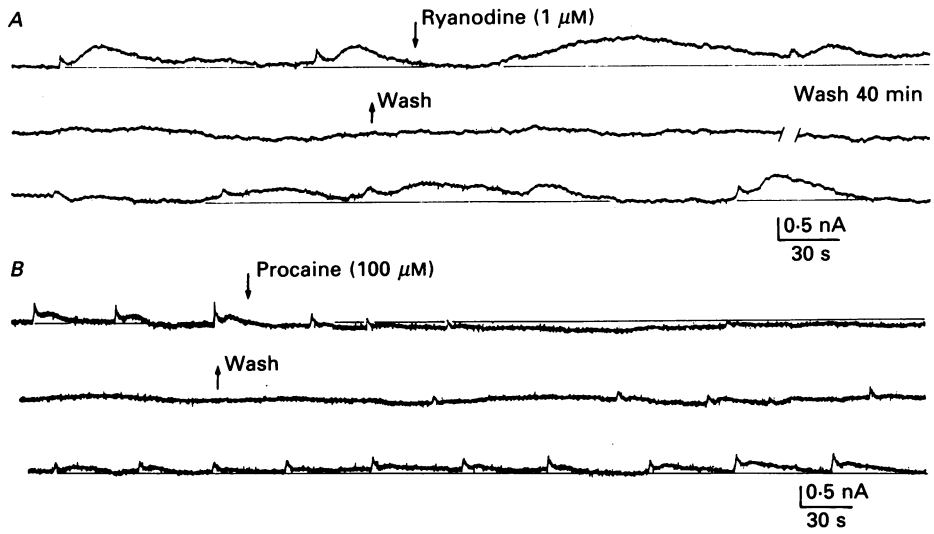

Fig. 3. Effects of ryanodine (*A*) and procaine (*B*) on I_{SH}. Data were obtained from different cells. Ryanodine (1 μM) and procaine (100 μM) were applied to the bath between downward and upward arrows. *A*, upper and middle traces represent consecutive recordings of the membrane current. Lower trace was recorded 40 min after removal of ryanodine from the superfusing solution. *B*, all traces were consecutive recordings of the membrane current. The holding membrane potential was −60 mV (*A*) and −63 mV (*B*).

Caffeine (1 mM) also triggered spontaneous oscillations of the holding current even in normally quiescent neurones. The effect of caffeine was reversible; the amplitude and the frequency of I_{SH} recovered 10 min after removal of caffeine from the superfusing solution. Another xanthine compound, 3-isobutyl-1-methylxanthine (IBMX, 1 mM), did not facilitate the frequency of the I_{SH}.

Ryanodine and procaine

It has been reported that ryanodine produces prolonged activation of calcium channels in sarcoplasmic reticulum, resulting in a depletion of the calcium store (Fleischer, Ogunbunmi, Dixon & Fleer, 1985; Meissner, 1986). Bath application of ryanodine (1 μM) for 5 min produced a transient outward current and blocked the appearance of I_{SH} ($n = 5$) in vesical pelvic neurones (Fig. 3*A*). The inhibition of I_{SH} lasted for more than 20 min even when ryanodine was removed from the superfusing solution; I_{SH} was partially restored within 40 min after withdrawal of ryanodine (Fig. 3*A*). Procaine has been reported to block calcium channels in sarcoplasmic reticulum, so that it blocks the release of calcium into the intracellular space (Endo, 1977). Treatment of vesical pelvic neurones with procaine (100–300 μM) caused a small inward current ($n = 3$). Procaine depressed the amplitude of I_{SH} and finally

blocked the occurrence of I_{SH} (Fig. 3*B*). The effect of procaine was readily reversible; I_{SH} was recovered within 15 min after removing procaine. In contrast, lidocaine (100–300 μM) had no effect on I_{SH} ($n = 4$).

Effect of lowering temperature

A quick lowering of temperature of muscle fibres produces a contraction in the presence or absence of caffeine due to the release of calcium from sarcoplasmic reticulum (Magaribuchi, Ito & Kuriyama, 1973; Sakai, Kurihara & Yoshioka, 1974). The release of calcium by a cold shock blocked the caffeine-induced hyperpolarization in neurones of bull-frog sympathetic ganglia (Kuba, 1980). In the rabbit pelvic neurones, lowering the temperature of the superfusing solution from 37·5 to 22·5 °C caused an outward current which reached a peak amplitude within 3 min and blocked I_{SH} ($n = 5$; Fig. 4*B*).

Separation of the slow I_{SH} from the fast I_{SH}

Tetraethylammonium (TEA) and barium

TEA blocks not only the delayed rectifier potassium current (Armstrong, 1975) but also some calcium-dependent potassium currents (Meech, 1972; Adams *et al.* 1982; Koketsu, Akasu & Miyagawa, 1982; Lancaster & Pennefather, 1987). Our previous data showed that the SH was partially reduced by TEA (Nishimura *et al.* 1988). Addition of TEA (5 mM) to the superfusing solution produced no significant change in the holding current but depressed the initial (fast) component of I_{SH} ($n = 3$; Fig. 5*A*). In contrast, the later (slow) component of I_{SH} appeared to be intact in the presence of TEA ($n = 3$; Fig. 5*A*). Extracellular barium has also been reported to block $g_{K,Ca}$ in autonomic neurones (Pennefather *et al.* 1985; Nishimura *et al.* 1988). Bath application of barium (1 mM) only completely reduced the rapid phase of I_{SH}, and not the slow I_{SH} ($n = 3$; Fig. 5*B*).

Apamin and (+)-tubocurarine

A previous current-clamp study has showed that apamin, a selective blocker for the slow $g_{K,Ca}$ (Pennefather *et al.* 1985; Kawai & Watanabe, 1986; Tanaka *et al.* 1986; Bourque & Brown, 1987), reduced the SH in pelvic ganglion cells (Nishimura *et al.* 1988). Figure 5*C* shows the effect of apamin on the calcium-dependent outward current under voltage-clamp conditions. Treatment of neurones with apamin (0·1–10 nM) caused no effect on resting conductance and holding current. Apamin (5 nM) completely depressed the initial component of I_{SH}, while it did not alter the slower component ($n = 3$; Fig. 5*C*). (+)-Tubocurarine is also known to block the $g_{K,Ca}$ in various neurones (Nohmi & Kuba, 1984; Dun, Jiang & Mo, 1986; Bourque & Brown, 1987; Nishimura *et al.* 1988). At a concentration of 100 μM, (+)-tubocurarine also selectively blocked the fast I_{SH} ($n = 3$; Fig. 5*D*). In contrast, the slow I_{SH} remained almost unchanged in the presence of (+)-tubocurarine (100 μM). The effects of apamin and (+)-tubocurarine were reversible when these drugs were removed from superfusing solution.

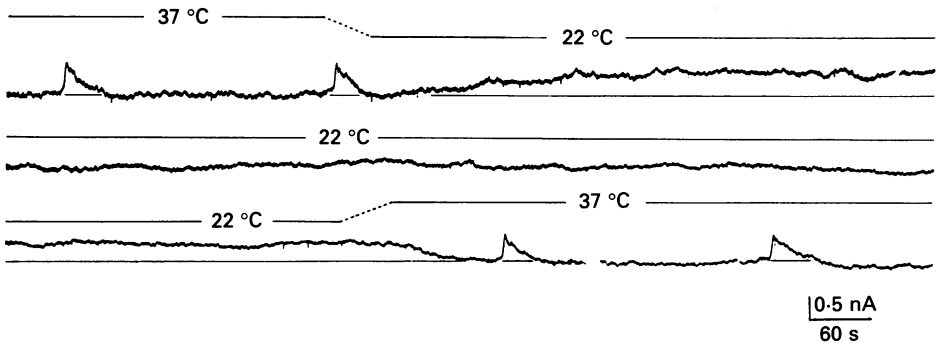

Fig. 4. Effect of lowering temperature on the I_{SH}. Temperature of the solution is shown between horizontal lines on each trace. Note that the occurrence of I_{SH}s was prevented at 22 °C. The holding membrane potential of the neurone was −65 mV.

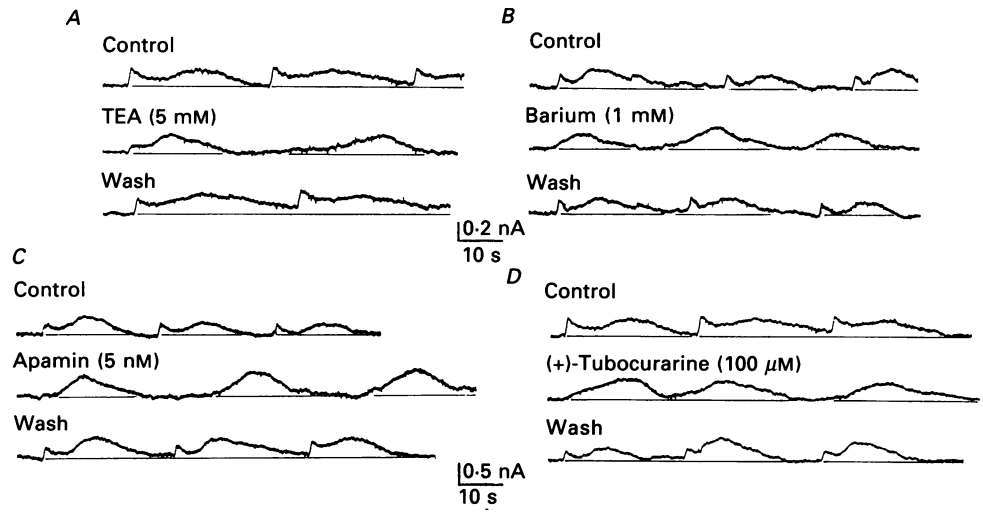

Fig. 5. Effect of TEA (*A*), barium (*B*), apamin (*C*) and (+)-tubocurarine (*D*) on the I_{SH} recorded from the same cell. *A* and *B*, all records were obtained from the same neurone held at −60 mV. Upper and middle traces were obtained respectively before and 10 min after application of Krebs solution containing TEA and barium. Lower traces were obtained 10 min after withdrawal of the drugs. Effects of apamin and (+)-tubocurarine on the I_{SH} were obtained from the same ganglion cell voltage clamped at −60 mV. Apamin (5 nM) and (+)-tubocurarine (100 μM) were added to the superfusing solution for 10 min. Upper traces were the control records of the I_{SH} obtained before application of apamin and (+)-tubocurarine. Middle and lower traces were taken during application and 30 min after removing these drugs, respectively. Note that these agents selectively blocked the initial rapid phase.

Injection of caesium ions

Intracellular caesium blocks potassium channels (Bezanilla & Armstrong, 1972; Gay & Stanfield, 1977; Tillotson & Horn, 1978). Figure 7*A* shows the fast and slow I_{SH}s recorded from a ganglion cell voltage clamped at −57 mV before injection of caesium ions. Caesium ions were then ionophoretically injected into the cell with

cathodal DC current (0·5–1·0 nA) in the current-clamp mode. Injection of caesium ions depolarized the membrane of pelvic ganglia neurones and prolonged the falling phase of the action potential (Nishimura *et al.* 1988; Akasu *et al.* 1990). The ganglion cells which contained caesium ions were again voltage clamped at the original

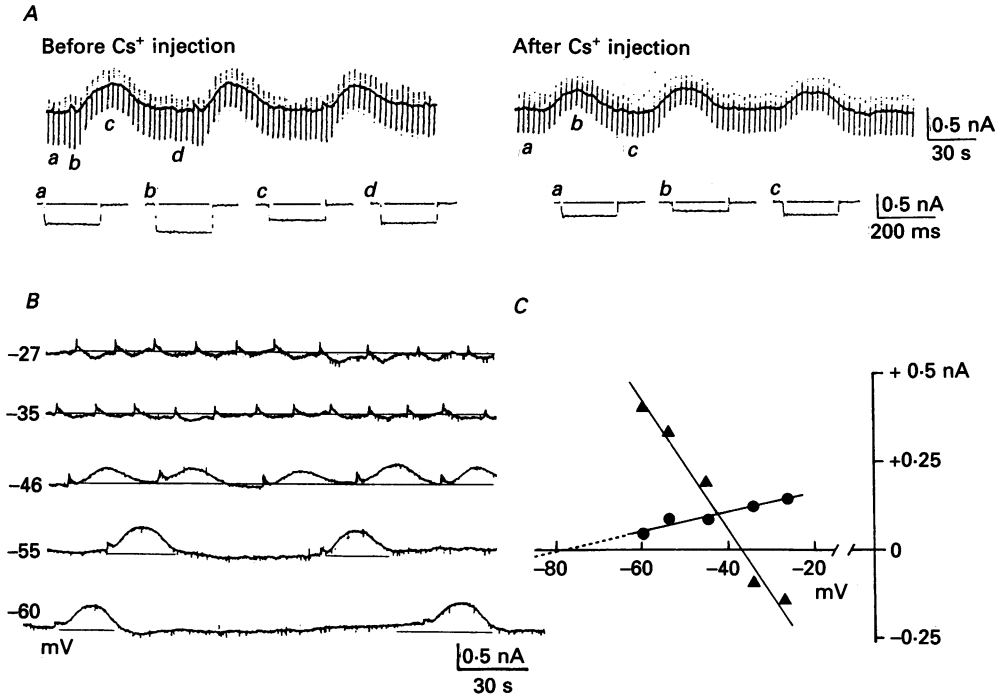

Fig. 6. *A*, I_{SH} obtained before (left) and 30 min after (right) injection of caesium. Control I_{SH} was recorded within 10 min after insertion of a microelectrode filled with 2 M-CsCl in Krebs solution. Vertical deflections are inward currents produced by hyperpolarizing voltage jump from −57 to −90 mV. Caesium injection was made by applying a depolarizing DC current with an amplitude of 0·5–1·0 nA for 10 min through the recording microelectrode under the current-clamp condition. The ganglion cell was again voltage clamped at −57 mV. The slow I_{SH}s were recorded 15 min after the termination of caesium injection. Records *a–d* shown in lower traces were expanded record of inward current taken at the time marked by respective letters in upper traces. *B*, the I_{SH} obtained from a single ganglion cell at different holding potentials between −27 and −60 mV. *C*, relationship between the amplitude of the I_{SH}s and the membrane holding potential. Data were from recordings of *B*. Circles and triangles indicate the amplitudes of the fast and slow I_{SH}, respectively. The dotted line is an extrapolation of the amplitude–voltage curve of the fast I_{SH}.

holding potential (−57 mV). The initial component of the I_{SH} was eliminated 30 min after injection of caesium ions, while the slow component of the I_{SH} was still observed in caesium-injected neurones ($n = 8$; Fig. 6*A*). The duration of the slow phase of I_{SH} was 31 ± 18 s in eight caesium-loaded neurones. The duration of the fast I_{SH}, estimated by subtraction from slow component, was 5 ± 3 s ($n = 8$).

Figure 6*A* also shows a change in the membrane conductance during the course of the isolated slow I_{SH}, where the fast component of the I_{SH} had been blocked by intracellular injection of caesium ions. The membrane conductance was measured by inward current evoked by a voltage jump from −57 to −72 mV (200 ms duration);

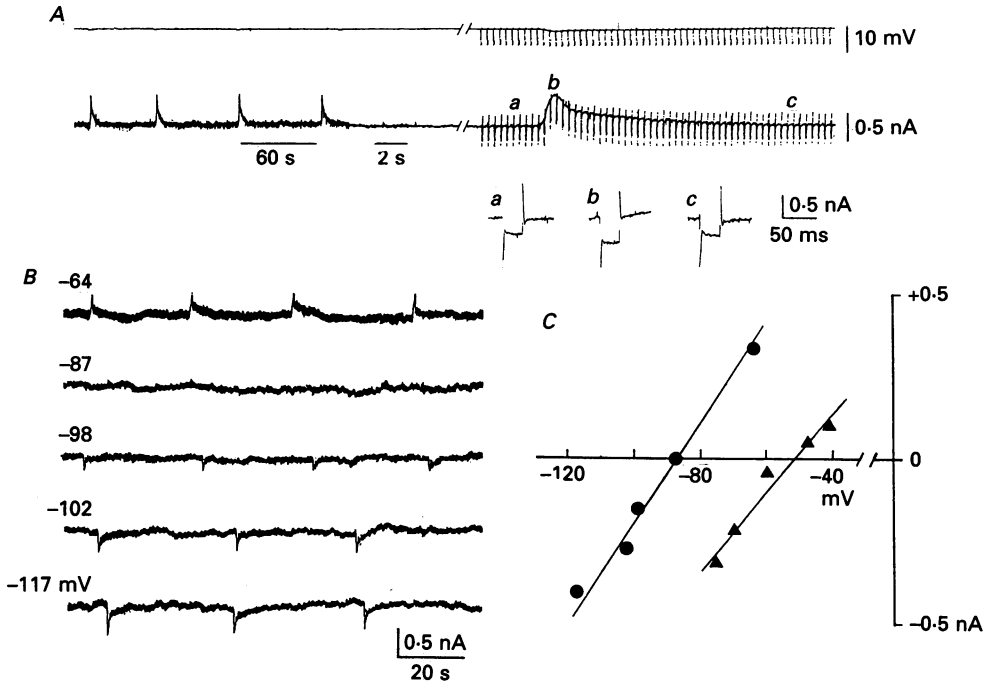

Fig. 7. *A*, conductance change during the fast I_{SH}. The ganglion cell was initially held at −64 mV. Hyperpolarizing voltage commands with a duration of 50 ms were applied at a frequency of 3 Hz to measure the membrane conductance. Expanded records of inward currents (*a–c*) in the lower trace were taken at the times marked by the respective letters in the middle trace. *B*, fast I_{SH}s recorded at various holding potentials. The external solution contained 2 mM-CsCl to block inward rectification. *C*, relationship between the membrane holding potential and the amplitude of the fast I_{SH}. Circles and triangles were obtained in the Krebs solution and high-K^+ (20 mM) solution, respectively.

it was decreased from 12±3 (n = 8) to 7±2 nS (n = 8) at the peak of the slow I_{SH} in caesium-loaded neurones (Fig. 6*A*). These results indicate that the I_{SH} is composed of two distinct current components: the fast I_{SH} accompanied by increased membrane conductance and the slow I_{SH} accompanied by decreased conductance.

Reversal potential of the fast I_{SH} and the slow I_{SH}

Figure 6*B* shows the relationship between the amplitude of I_{SH} and the holding voltage. The fast I_{SH} increased its amplitude at depolarized membrane potentials and decreased at hyperpolarization. Conversely, the slow I_{SH} decreased its amplitude and finally reversed polarity at a depolarizing holding potential. Figure 6*C* shows the relationship between the holding voltage and the peak amplitudes of the initial phase

and slow phase of I_{SH}s. The slow I_{SH} reversed at −37 mV (−30±6 mV, $n = 4$). This was close to the equilibrium potential for chloride ions (Tokimasa, Nishimura & Akasu, 1988; Akasu *et al.* 1990). The reversal potential of the fast I_{SH}, obtained by extrapolation of the amplitude–voltage curve, was −78 mV (−80±5 mV, $n = 4$; Fig. 6*C*).

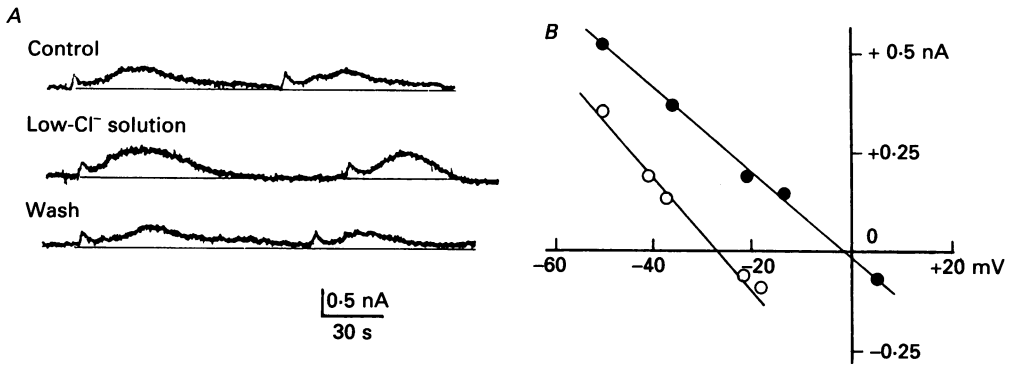

Fig. 8. *A*, the fast and slow I_{SH} obtained from a ganglion cell in low-chloride solution. The holding potential was −58 mV. Control record (upper trace) was obtained in Krebs solution before application of low-chloride solution. The low-chloride solution was made by substitution of 67 mM-isethionate for equimolar chloride. Middle trace was obtained after application of the low-chloride solution for 10 min. *B*, relationship between peak amplitude of the slow I_{SH} obtained in Krebs solution (○) and low-chloride (68 mM) solutions (●).

The reversal potential of the fast I_{SH} was also examined in neurones where I_{SH} had no slower current component (Fig. 7*B*). The duration of the fast I_{SH} was 4±2 s ($n = 8$), comparable to that of the fast I_{SH} obtained by subtraction of the slow I_{SH} from the total I_{SH} (see above). The fast I_{SH} was reversibly blocked by apamin (1 nM), TEA (5 mM) and barium (1 mM) (not shown). The fast I_{SH} was associated with an increase in the membrane conductance (Fig. 7*A*). The amplitude of the fast I_{SH} decreased and finally reversed polarity at −87 mV, when the neurone was hyperpolarized (Fig. 7*B*). Figure 7*C* shows a linear amplitude–voltage relationship of the fast I_{SH}. From four other neurones, the reversal potential of the fast I_{SH} was −88±7 mV. The reversal potential of the fast I_{SH} shifted to −51 mV when the concentration of extracellular potassium ions was increased to 20 mM (Fig. 7*C*). These values are in agreement with the equilibrium potential for potassium ions calculated from the Nernst equation (see also Nishimura *et al.* 1988).

Chloride dependence of the slow I_{SH}

Figure 8*A* shows the effect of lowering extracellular chloride activity on I_{SH}. Substitution of the impermeable anion isethionate (67 mM) for chloride augmented the amplitude of the slow I_{SH}, without changing the fast I_{SH} (Fig. 8*A*). The reversal potential of the slow I_{SH} was again examined in a low-Cl^- Krebs solution. Figure 8*B* shows an example of these experiments. The reversal potential of the slow I_{SH} shifted to −2 mV (mean, +1±8 mV, $n = 5$) in low-Cl^- solution.

A possible chloride-dependent mechanism for the slow I_{SH} was also investigated using the stilbene derivative, SITS. This compound has been reported to block chloride currents in several excitable tissues (Gray & Ritchie, 1986; Inoue, 1986; Bader, Bertrand & Schlichter, 1987; Bretag, 1987; Korn & Weight, 1987; Akasu

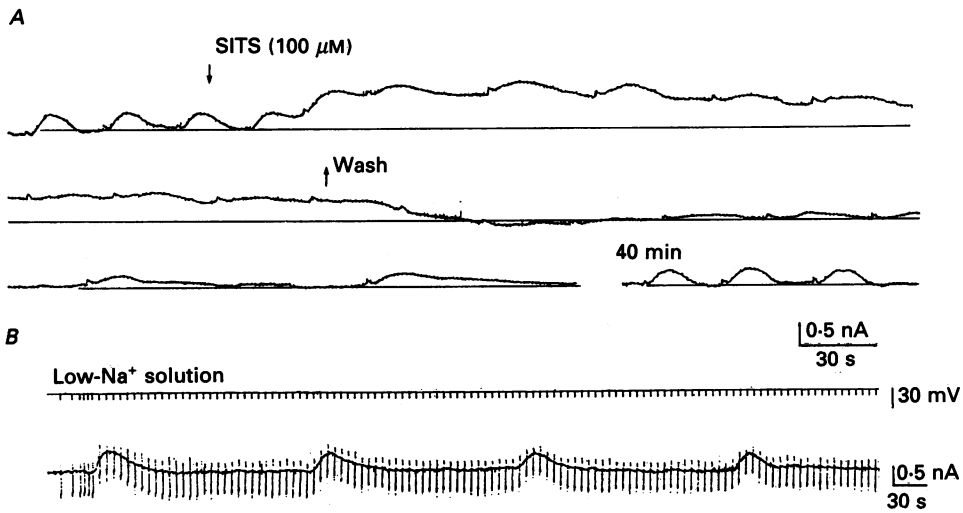

Fig. 9. *A*, effect of SITS (100 μM) on the I_{SH}. Upper, middle and lower left traces are consecutive recordings of fast and slow I_{SH}s. The holding potential was −62 mV. SITS (100 μM) was added to the superfusing solution between the downward and upward arrows. The lower right record was obtained 40 min after removal of SITS from the external solution. Horizontal lines indicate initial current level. *B*, slow I_{SH} recorded in a low-sodium (26·2 mM) solution, where sodium (117 mM) was replaced with equimolar TEA.

et al. 1990). Bath application of SITS (500 μM) caused an outward shift of the holding current associated with a decreased membrane conductance. The slow I_{SH} was gradually depressed in the presence of SITS ($n = 3$; Fig. 9*A*). When the slow I_{SH} was blocked, the fast component of the I_{SH} was still observed in the presence of SITS. The effect of SITS was reversible, although the depression of the slow I_{SH} lasted for more than 40 min in recovery solution.

It has previously been reported that an increase in intracellular calcium produces a long-lasting outward current as a result of blockade of the resting sodium conductance in bull-frog sympathetic ganglia (Akaike & Sadoshima, 1989). Experiments were therefore conducted to determine whether the slow I_{SH} is carried primarily by sodium ions. In caesium-loaded neurones, however, a slow I_{SH} with amplitudes ranging between 0·1 and 1 nA ($n = 7$) could be recorded in a low-sodium Krebs solution, where 117 mM-sodium chloride was replaced with equimolar TEA chloride (Fig. 9*B*). Substitution of choline (117 mM) for external sodium also produced no significant effect on the slow I_{SH}. In addition, tetrodotoxin (3 μM) did not depress I_{SH} (see also Nishimura *et al.* 1988). These data do not support the notion that the slow I_{SH} is mainly carried by sodium ions in pelvic neurones.

Contribution of I_{SH} to the spike after-hyperpolarization

It has been reported that an influx of calcium ions during the action potential activates not only the spike after-hyperpolarization (AHP) but also the SH (Kuba, 1980; Nishimura *et al.* 1988). We evaluated the contribution of a calcium-dependent

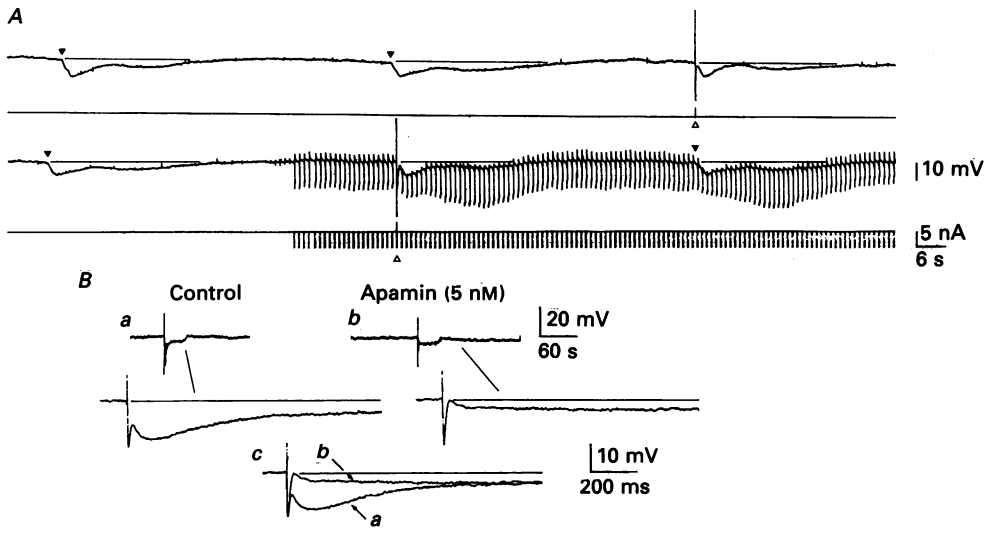

Fig. 10. *A*, spontaneously occurring SH (▼) and SH evoked by an action potential (△). The resting membrane potential was −63 mV. Upper and lower traces indicate membrane potential and current, respectively. *B*, effect of apamin (5 nM) on the spike after-hyperpolarization and SH evoked by a single action potential. Upper records *a* and *b* were obtained before and 10 min after application of apamin (5 nM), respectively. Expanded records of action potentials of *a* and *b* were shown in middle traces. In record *c*, traces *a* and *b* were superimposed.

chloride current to the spike AHP. Figure 10*A* shows sample recordings of spontaneously occurring SH and the SH triggered by an action potential. A single action potential evoked by a depolarizing current pulse was followed by a fast AHP comparable to I_C in sympathetic neurones (Adams *et al.* 1982; Nishimura *et al.* 1988). The spike AHP was also followed by an additional hyperpolarizing after-potential with two components (Fig. 10*B*). The time course and membrane resistance changes of the two phases of hyperpolarization were similar to those of the spontaneously occurring SH. Figure 10*B* shows the effect of apamin (5 nM) on the spike after-potentials. Apamin did not affect the fast AHP. On the other hand, the initial (fast) component of the evoked SH was strongly depressed by apamin. The latter component of SH appeared to remain in the presence of apamin (Fig. 10*B*).

We also examined the contribution of chloride current to the outward current underlying the AHP (I_{AHP}) following an action potential. I_{AHP} could be detected after the termination of brief (25–50 ms) but large (50–90 mV) step depolarizations from a holding potential close to the resting potential (Fig. 11). I_C could not be recorded, because of the slow speed and low gain of the single-electrode voltage-clamp procedure used in the present study. I_{AHP} was associated with increased

membrane conductance during its initial phase but a decrease conductance was seen at a later phase (Fig. 11*A*). When ganglion neurones were superfused with a Krebs solution containing nominally zero calcium and 12 mM-magnesium, I_{AHP} was completely depressed (Fig. 11*B*), suggesting that I_{AHP} is produced by activation of

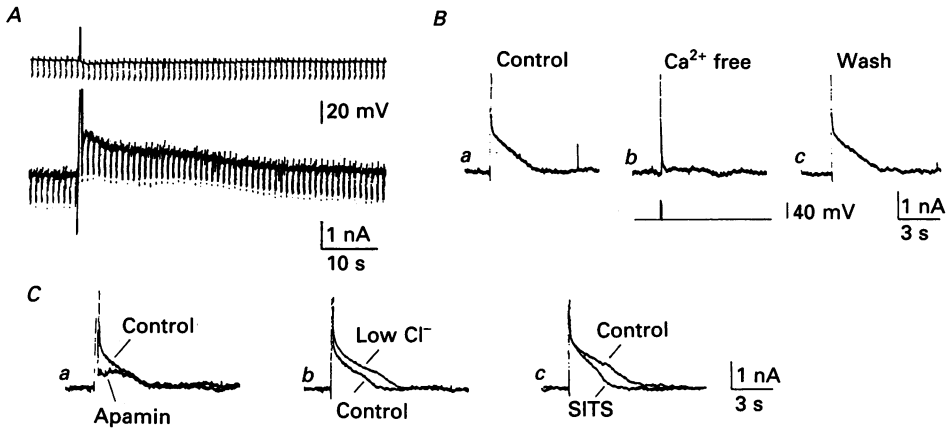

Fig. 11. *A*, I_{AHP} evoked by a depolarizing step command (50 ms, 60 mV) from a holding potential of −62 mV. Hyperpolarizing voltage commands with a duration of 200 ms were applied to measure the membrane conductance. *B*, effects of removing calcium on I_{AHP}. Calcium-free solution contained nominally zero calcium and 12 mM-magnesium. *C*, effects of apamin (*a*), low chloride (56 mM) (*b*) and SITS (100 μM) (*c*) on the I_{AHP}. SITS was applied to the bath for 10 min.

calcium-dependent conductance(s). In the presence of apamin (5 nM), although the I_{AHP} was significantly depressed, the later component of I_{AHP} still appeared to be present ($n = 4$; Fig. 11*C*). Lowering external chloride concentration increased both the amplitude and the duration of I_{AHP} (Fig. 11*C*). SITS (100 μM), a chloride channel blocker, depressed the later portion of the I_{AHP}; as a result, the duration of I_{AHP} was depressed by SITS (Fig. 11*C*).

DISCUSSION

The results of the present study have demonstrated rhythmic oscillations of membrane current (I_{SH}) occurring at fairly constant intervals (30 s to 5 min) in neurones of vesical pelvic ganglia. Several lines of evidence suggest that I_{SH} is regulated by increased intracellular calcium concentration. (1) The removal of extracellular calcium blocked I_{SH}. (2) Caffeine increased the frequency of I_{SH}. (3) I_{SH} was inhibited by lowering the temperature of the superfusing solution. (4) Procaine and ryanodine blocked the appearance of I_{SH}. Ryanodine has been reported to shorten the spike AHP by activating calcium-dependent calcium release in rat sympathetic neurones (Kawai & Watanabe, 1989). In rabbit vesical pelvic neurones, ryanodine also depressed the duration of the spike AHP (T. Nishimura, T. Akasu & T. Tokimasa, unpublished observation), suggesting that a similar mechanism contributes to the generation of I_{SH}. (5) Our previous study showed that intracellular

injection of EGTA blocked the occurrence of the SH (Nishimura *et al.* 1988). Rhythmic oscillations of the resting membrane potential generated by periodic release of calcium from an intracellular store site have been demonstrated in various neuronal tissues (Kuba & Nishi, 1976; Suzuki & Kusano, 1978; Kuba, 1980; Morita *et al.* 1982; Kuba *et al.* 1983), smooth muscles (Benham & Bolton, 1986; Ohya, Kitamura & Kuriyama, 1987; Hume & Leblac, 1989) and parotid acinar cells (Gray, 1988).

Blocking agents for $g_{K,Ca}$ (apamin and (+)-tubocurarine) (Nohmi & Kuba, 1984; Pennefather *et al.* 1985; Dun *et al.* 1986; Kawai & Watanabe, 1986; Tanaka *et al.* 1986; Bourque & Brown, 1987; Nishimura *et al.* 1988) inhibited the initial (fast) component of the I_{SH}, without affecting the later (slow) I_{SH}. The reversal potential of the isolated fast I_{SH} was near the equilibrium potential for potassium ions (see also Nishimura *et al.* 1988). Thus, the fast I_{SH} in vesical pelvic neurones appeared to be produced by an activation of $g_{K,Ca}$. The reversal potential of the initial component of the I_{SH} was, however, about 10 mV less negative than that of the isolated fast I_{SH} (Figs 6 and 8). This difference may reflect that the slow I_{SH} can co-exist with the fast I_{SH} during the initial phase of the I_{SH}.

An additional calcium-dependent ionic conductance, different from the $g_{K,Ca}$, appears to contribute to the slow I_{SH}. (1) The slow component of I_{SH} was associated with a decrease in the membrane conductance. (2) The slow I_{SH} was resistant to blockers of the calcium-activated potassium channels, apamin, (+)-tubocurarine, TEA and barium. (3) Intracellular injection of caesium ions inhibited the fast I_{SH} but not the slow I_{SH}. (4) The slow I_{SH} was still recorded in a low-sodium (26·2 mM) solution. (5) The slow I_{SH} reversed its polarity at potentials near the equilibrium potential for chloride ions (Tokimasa *et al.* 1988; Akasu *et al.* 1990). (6) Lowering extracellular chloride increased the amplitude of the slow I_{SH} and produced a positive shift of the reversal potential. (7) SITS, a blocker for chloride channels (Inoue, 1986; Gray & Ritchie, 1986; Bader *et al.* 1987; Bretag, 1987; Korn & Weight, 1987; Akasu *et al.* 1990), inhibited the slow I_{SH}. These results suggest that the slow I_{SH} is due mainly to the inhibition of a calcium-dependent chloride conductance.

It has been reported that a voltage-dependent calcium influx activates chloride channels, producing the slow inward tail current in several neurones (Owen, Segal & Barker, 1984; Mayer, 1985; Scott, McGuirk & Dolphin, 1988; Akasu *et al.* 1990). The chloride-dependent tail current in vesical parasympathetic neurones was, however, voltage dependent; it activated at potentials more positive than −30 mV (Akasu *et al.* 1990). The calcium-activated chloride current does not appear to contribute to the slow I_{SH}, because the I_{SH} does not depend on the holding voltage. The chloride tail current was not involved in the AHP and SH triggered by a single action potential, because the chloride tail current was not significantly activated by depolarizing voltage commands with durations shorter than 100 ms (Akasu *et al.* 1990). Furthermore, the spike after-depolarization produced by activation of calcium-dependent chloride current appeared only when the ganglion neurones fired action potentials repetitively (Akasu *et al.* 1990). Recently, it has been reported that voltage-insensitive calcium-dependent chloride conductance is present in frog dorsal root ganglion cells (Inoue, Oomura, Yakushiji & Akaike, 1986). During I_{SH}, this resting voltage-independent chloride current may be depressed by calcium in vesical

pelvic ganglion cells. The present study did not investigate how intracellular calcium regulates two different conductance systems, potassium and chloride conductances with fast and slow kinetics. A second messenger might be involved in a process for activation of the caffeine-induced long-lasting current (Pfaffinger, Leibowitz, Subers, Nathanson, Almers & Hille, 1988; Akaike & Sadoshima, 1989).

A single action potential was followed by a spike after-hyperpolarization which is produced mainly by activation of $g_{K,Ca}$ in the rabbit pelvic ganglion neurones (Nishimura *et al.* 1988). The present study has shown that the action potential also triggered a long-lasting hyperpolarizing response with time course and conductance change similar to those of the SH. The triggered SH was composed of fast and slow hyperpolarizations associated with decreased and increased membrane conductances, respectively. The latter hyperpolarization was insensitive to apamin. The I_{AHP} also had a slow current component which was not blocked by apamin. Lowering the extracellular chloride concentration (56 mM) prolonged the I_{AHP} duration, and increased the amplitude of its slow component. In contrast, the duration of I_{AHP} was depressed by SITS. It is, therefore, suggested that calcium-dependent chloride current also contributes to the I_{AHP} after an action potential.

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